

Building an umbrella for 1985

Next month's meeting between the United States and the Soviet Union is welcome. But the meeting will best succeed if its goals are modest, a standing commission on strategic arms in the first place.

NEXT year will get off to an interesting start. On 7 January, Mr George Shultz, the US Secretary of State, and Mr Andrei Gromyko, the Soviet Foreign Minister, are due to meet in Geneva to take up again the issues of arms control whose discussion was abruptly halted just over a year ago, when the Soviet Union walked out of the Geneva talks on intermediate-range missiles (INF) and strategic missiles (START). What is the chance that next month's meeting will make progress when so much has so recently gone wrong?

Three separate sets of auguries suggest that, this time, the two superpowers will have an incentive to agree. First, military costs are hurting both the superpowers; in the United States, President Reagan's hope of keeping the federal deficit within reasonable bounds seems yet again destined to be frustrated by Mr Caspar Weinberger's demands, while the Soviet government has signalled the high cost of defence by increasing the nominal proportion of its budget devoted to military affairs. Second, there has been a change of leadership in Moscow but also in Washington; Andropov has been succeeded by Chernenko and, just as significant, first-term Reagan, preoccupied with re-election, has been succeeded by second-term Reagan, likely (in the manner of his predecessors) to be anxious about what the history books will say of him. Then, third, both superpower governments have a common interest in reaching some tangible agreement on arms control before the third review conference of the Non-Proliferation Treaty fixed for next August; if they have nothing to show for their obligation to negotiate on strategic arms, they will earn another thorough drubbing from the non-nuclear signatories of the treaty, the continuation of which could easily be jeopardized. So something may emerge.

What? To begin with, not much. The announced objective of the Shultz-Gromyko talks is to find an umbrella under which serious negotiations may later take place. They are to be talks about talks, not substance. But that does not imply that nothing can be decided. What stands out from the experience of the past quarter of a century (since the Partial Test-Ban Treaty, signed in 1963 after five years of desultory negotiations) is that the pace of military development is too fast for the mechanisms of diplomacy. If the Soviet Union had not walked out of INF and START, at the end of 1983, in protest against the long-advertised deployment of intermediate-range missiles in Western Europe, President Reagan's star-wars speech in March this year might well have made them pack their bags.

Commission

So the obvious and urgent need, and the best kind of umbrella, is for a mechanism for the avoidance of surprises. Negotiations on particular measures of arms control are important, necessary and, indeed, long overdue. But there also needs to be a mechanism by which the superpowers can avoid startling each other, triggering off by either a walk-out from some continuing negotiation (at best), perhaps a more tangible hostile action. One of the benefits of ratifying the SALT II Treaty (signed in 1979) is that there would by now have been a standing commission representing both the superpowers to superintend the working of that agreement. The immediate objective, for Messrs Shultz and Gromyko, should be to establish just such a commission, but with wider terms of reference. There is no reason why an agreement to that effect

should not be realized in the first week of next year, or why a working commission should not be a going concern by the end of January.

But will there not be endless haggling about the terms of reference of such a body? There could, but need not, be. The practical needs are obvious. A standing commission (with an agreed timetable of frequent meetings) should be a forum in which one side or the other could disclose and explain strategic plans that would otherwise be surprising threats. Even the star-wars speech would have been less an irritant of Soviet susceptibility if the United States had been compelled to describe its plans in advance, explaining that, for the time being, star wars is a five-year programme of research, not even a development programme. A standing commission would also be a way of raising in private allegations that existing agreements or understandings are being violated. The United States has needlessly soured the international climate in the past year with public accusations that the Soviet Union has violated the unratified Threshold Test-Ban agreement (with another underground explosion marginally on the 150,000 tonne of TNT equivalent only a month ago), but has a legitimate question to ask about the phased-array radar being built in central Siberia, apparently in violation of the Anti-Ballistic Missile Treaty, signed in 1972, which allows such instruments only near a superpower's borders.

Modest goals

The chief practical value of a standing commission, however, would be as a means of agreeing in advance which specific strategic agreements are attainable. Part of the difficulty in the past decade is that the superpowers have locked themselves into over-ambitious negotiations. Although the breakdown of the Comprehensive Test-Ban Treaty in 1979 was at the time attributable to technical factors centring on verification, the real trouble was that neither US nor Soviet generals were ready for such a step. Nor are they now. Even the INF and START negotiations, which at various times in 1982 and 1983 looked hopeful, would have asked a lot of the generals and the US Senate, which would have had to ratify whatever agreements finally emerged. Far better, in these changed circumstances, that the two superpowers should informally decide privately on a sequence of modest goals that can then be quickly negotiated. It would be a tonic for everybody if there could be a succession of agreements on strategic (not always nuclear) weapons, however modest.

The ready objection to arms control by instalments along these lines is that its consequences will be undramatic. The non-nuclear signatories would for example spend next August/September complaining that nothing substantial had been achieved. Almost certainly, the Soviet Union will be looking for a quick dramatic agreement from beneath the umbrella now to be erected, if only to make plain that it has lost no face by walking out at Geneva a year ago, but also from a genuine if misguided belief that the United States is about to populate interplanetary space with novel and dangerous weapons. (That is at least five years and another presidential election away.) But a little reflection will show that even an attempt to return to INF and START would be imprudently ambitious but also intrinsically insufficient. Eighteen months of hard negotiation got nowhere and then, by



collapsing, did more harm than good; yet even these parallel negotiations were recipes for letting important issues (British and French nuclear weapons, the sense in Western Europe and the Soviet Union that intermediate-range weapons can have a strategic function) fall between the cracks. In retrospect, even unratified SALT II looks over-ambitious or, at least, too complicated to be workable.

So is there nothing to be done? Far from it. For starters, why not agree to ratify the existing treaties on the maximum size of underground explosions and that which outlaws peaceful nuclear explosions except with advance notice and inspection by the other side? So much could be agreed next month. So too could be the principles of a treaty to ban the testing of anti-satellite weapons. The United States, which has made one test with an air-launched rocket, says the Soviet Union has already deployed such a system, but its performance is known to be so poor as to be irrelevant. The next best bet would be an attempt to define some of the untidy edges of the aborted INF and START negotiations, the manner in which bomber aircraft should be traded off against missiles of long or shorter range, for example, or the relevance of battlefield weapons to the strategic balance.

Haggling of that kind, however necessary, will cut very little ice with the non-nuclear members of the Non-Proliferation Treaty, who will be looking for limitations, even reductions, of strategic nuclear weapons and, now, assurance that the region beyond the atmosphere will not become a battlefield. They will not have either by next August. But what they could be offered is a promise to work towards a much simpler agreement than either SALT II or the kinds of treaties likely to have emerged from INF and START. Why not simply settle for a limit on the number of nuclear weapons (separate warheads) that each side may retain, letting it decide for itself how they should be deployed? Verification is the obvious difficulty, which could be overcome by a necessarily private exchange of information on past production of fissile material. There might then follow an agreement to a cut-off on military production of fissile material, a scheme once dear to the French. And if the limit on nuclear warheads were small enough, the case for the deployment of whatever emerges from the star-wars research would melt away. Is all that too much to hope for? By recent experience, it is too much. The crucial question, which may be answered next month, is whether Mr Shultz and Mr Gromyko, and their political masters, will be able to change the tone of arms control. □

Agency two-step hazard

Trading the White House science office for a Department of Science would be wrong.

FEW things give a President of the United States such a feeling of accomplishment, with so little actual accomplishment, as a reorganization of the executive branch. Bureaux become departments, offices are merged, titles appear and disappear, but nothing changes. It is a generally harmless exercise, one that gives the illusion of increased efficiency while keeping the chief executive too busy to make a genuine nuisance of himself.

But there is something else going on with the proposals leaking out about President Reagan's plans for reorganization. Presidential science adviser George Keyworth is one of those suddenly enamoured of a cabinet-level Department of Science, apparently undeterred that this idea has been proposed more than a hundred times in the past twenty years, according to the Congressional Research Service. The proposed Department of Science would assemble under it all the research agencies of the federal government except agriculture, defence and those inextricably bound to a regulatory function, which means the Environmental Protection Agency and the Food and Drug Administration. So its components would include the National Science Foundation (now an independent agency), the National Bureau of Standards and the National Oceanic and Atmospheric Administration (which have always been somewhat out of place in the Department of Commerce), the National Institutes of Health, the

research components of the National Aeronautics and Space Administration (NASA) and of the Department of Energy (which department the Reaganites want anyway to eliminate) and the US Geological Survey.

Meanwhile, the murmurs are growing about plans drastically to reduce or even eliminate the Office of Science and Technology Policy (OSTP), the science adviser's office. The uncharitable (and probably wrong) interpretation is that Keyworth does not want any rivalry when he becomes the first Secretary of Science. A more likely interpretation is that even a vaguely independent office is a threat to the finely tuned political operations of the White House.

Although the idea of a Department of Science is probably less political (in the bad sense of the word) in origin, it is equally troublesome. And it is emphatically not in the category of cosmetic reorganizations. It has major consequences, not all of them good. To be sure, a cabinet-level department would, as they say in Washington, enhance the "visibility" of science; while OSTP, as an advisory body, can simply be ignored (and would be without the personal rapport that Keyworth apparently enjoys with Reagan), a Department of Science would have to be listened to in budget deliberations. And surely it would be better for science to have science budgets reviewed in the context of science budgets, not weighed against other pet projects that an agency may be pursuing. This would be especially true for space science, which must now compete within NASA against monstrosities such as the space station, created by NASA principally to perpetuate its institutional existence.

But the dangers are enormous. Unaesthetic though the present hodge-podge of science agencies may be, it serves a very real purpose in allowing expression for a diversity of views for the support of science. Putting them all together would render the entire federal research and development budget vulnerable to the sort of short-sighted political manipulations that, hitherto, have mercifully not afflicted more than one agency at a time. (Remember Research Applied to National Needs?) Worse, a cabinet-level department would be a perfect political plum for a political hack. Scientists may not always make good administrators, but they tend not to go far with half-baked schemes. The tradition of putting scientists in charge of science agencies has on the whole proved sound.

As for OSTP, it has obviously become a target for the political cosmeticians in the White House who ensure that the television cameras are always in the right place at the right time and that reporters are not given the opportunity to ask questions to which the President does not know the answers. As exceedingly careful as Keyworth has been to back the party line, even when that has meant making dubious statements about star wars and space stations, he is nonetheless regarded as an unnecessary risk by those who want all decisions made on the basis of political appearance. Similar reasoning explains the ambition to eliminate the Council of Economic Advisors, which has been on the hit list ever since Martin Feldstein dared to suggest that \$200,000 million deficits might have something to do with high interest rates. (The Council on Environmental Quality was all but eliminated four years ago, when the entire staff was fired and replaced by political familiars from Reagan's time as governor of California.)

OSTP, as now constituted, strikes a reasonable balance between window-dressing at one extreme and centralized control of federal research (which is what a Department of Science would do) at the other. The critics are right when they say it is weak because it is only advisory; that is what it should be.

Many who depend on one agency or another for their livelihoods will predictably oppose on narrow grounds any changes in the *status quo*. But they need not apologize for entertaining serious reservations about the proposal for a Department of Science, which should (and probably will) end up in the same place as it has on the 99 previous occasions it has surfaced. But the worrying aspect of these proposals is that they appear to be independent. OSTP could die an early death, and the plan for a Department of Science a lingering death in Congress. And then there would be nothing. □

Nuclear winter

US National Academy urges greater caution

Washington

A NATIONAL Academy of Sciences panel has concluded that* while the "nuclear winter" scenario — recently popularized by Carl Sagan — is a "clear possibility", the uncertainties are so great as to make it impossible to "put a number on the temperature changes or other climate effects". George Carrier of Harvard University, chairman of the panel, said he believed that both Sagan and Edward Teller, his most prominent critic, have been taking the results of recent calculations of the climatic effects of nuclear war "too literally", and that current atmospheric models can at best give "indications" of the magnitude of the global cooling that might follow an all-out nuclear exchange.

In testimony to Congress and in repeated public statements, Sagan has raised the



possibility that the fall in temperature following a nuclear war would be so great (37 degrees centigrade, according to his model calculations) and of such duration (several months or longer) that the survival of the human race would be threatened.

The panel's calculations produced similar results — a cooling of 20 to 25 degrees centigrade in the Northern Hemisphere, lasting for 6–20 weeks. But throughout their report, the panel stressed that uncertainties in both the assumptions and the models could vastly alter these numbers. The major uncertainty identified by the panel is the quantity of smoke that would be generated in fires set off by nuclear blasts. Small smoke particles are very efficient absorbers of solar radiation; the panel estimated that 180 million tonnes of smoke would be produced in a nuclear war that involved half the world's nuclear arsenal, and that more than 90 per cent of the incoming sunlight would be absorbed as a result. But the plausible range of smoke emissions is great, from 20 to 650 million tonnes; and because of the exponential relationship that determines total absorption, the light loss would diminish rapidly as the amount of smoke drops below 40 or 50 million tonnes.

Even with additional research, the panel

acknowledges, the climate effects of nuclear war will not be defined with great precision in the next few years. Indeed, because many factors depend on human decisions that can be changed at will, any calculation involves a significant measure of irreducible uncertainty.

The panel raised several apparently new questions about the nuclear winter phenomenon. One is the seasonal variations. The panel's global-circulation model calculations suggest that the effect would be much

Uncertainties of climatic change

THE academy report on nuclear winter studiously avoids the use of that term, even in quotation marks. The bulk of the report is concerned with the analysis of the assumptions on which calculations of the climatic effects of the nuclear war have been based. The committee has not constructed climatic models of its own, but has persuaded climatologists to re-run their models with different assumptions.

The committee's starting assumption is that 6,500 megatons of Soviet and US nuclear weapons would be detonated in northern mid-latitudes (compared with the 5,000 megatons in the Turco *et al.* baseline case). This hypothetical war involves warheads yielding 1.5 megatons or less, directed at hardened missile silos (one ground-burst each) and at economic targets (mostly in populated centres). But one of the variants considered includes an extra 100 20-megaton explosions.

For the study's baseline case, the quantity of the fine sub-micrometre dust carried into the stratosphere works out at 15 million tonnes (but could be twice as much), rather less than that calculated by Turco *et al.* But the addition of 20-megaton explosions makes it credible that 1,000 million tonnes of fine dust might be lofted there.

The academy study agrees with the conclusion of Turco *et al.* that soot carried into the stratosphere from fires ignited by nuclear explosions would have a more marked effect than dust on the flux of solar radiation absorbed in the atmosphere, chiefly because of the abundance of particles with sizes of the order of 0.1 micrometres in the smoke from burning fires.

The most important part of the report is the committee's careful discussion of the variables affecting smoke production, together with its review of the observations (from forest fires, for example) leading to the view that the size-distribution of the particles is log-normal. The conclusion is

greater in the summer than in the winter. And models of spring and summer conditions also suggest that a large transport of smoke particles across the Equator could occur in those seasons, spurred by solar heating of the debris cloud.

The panel's study was commissioned by the Department of Defense early in 1983, several months before Sagan publicized his findings. Although the report was completed almost a year ago, it went through an arduous review process before its release last week. A number of reviewers had strongly opposed the inclusion of any numerical results, no matter how strongly qualified, arguing that they would be misinterpreted. Indeed, several newspaper accounts reported the academy as having given its "seal of approval" to Sagan's conclusions.

Stephen Budiansky

that in the baseline case, 180 million tonnes of smoke would be produced (five-sixths of it from urban fires), that most of this would be distributed in the lower 9 kilometres of the troposphere and that very little would be injected into the stratosphere.

In the committee's baseline case, 250,000 square kilometres each of forest and urban landscape would be burned, with urban areas yielding 100 times as much smoke (3 g cm^{-2}) as forest. Uncertainty stems from possible variations not merely in smoke loading but also in the optical properties of the smoke particles and, in particular, the efficiency of the particles in absorbing visible radiation. The committee says that it cannot combine its estimates of uncertainty due to separate parameters because so little is known of the dispersion of the various sources of error.

Among the sources of error in the model calculations of the climatic effects of these smoke loadings, the committee singles out the impossibility, as things are, of incorporating into climatic models "the cloud microphysical processes that are primarily responsible for the removal of particulates from the atmosphere".

The results of the modelling studies carried out for the committee are qualitatively similar to those of Turco *et al.* Briefly, in the first few days after a nuclear exchange on the scale assumed, the Northern Hemisphere would contain several patches above which the optical depth of the smoke-laden atmosphere would exceed 20 (where the optical depth is the natural logarithm of the ratio of incident and transmitted intensities). Temperatures beneath the cloud of smoke would indeed be sharply decreased, in the worst case by a maximum of 35 degrees centigrade over six months but, with different assumptions about the speed with which smoke would be removed by rainfall, by a maximum of 15 degrees centigrade and to a substantial extent over a period of a month. □

The effects on the atmosphere of a major nuclear exchange. National Academy Press, Washington, DC, 1985, \$14.50.

Japanese universities

Private sector under new stress

Tokyo

JAPAN's private universities are making plans to get rid of their reputation for profligacy and mismanagement by voluntarily opening their account books to the public. Their poor image has recently cost them dear — three years of cuts in government subsidies have pushed more than half of them into debt and are forcing them to cut back on facilities and scholarships.

At present, Japanese law does not require private universities publicly to declare the source of their income or how it is spent, and much of their financial management has been veiled in secrecy. What has become visible has often been the result of a scandal: universities have faked reports of their expenses in order to receive larger subsidies, have taken huge "donations" to bend the admission rules for sub-standard students and have paid excessive salaries to directors and professors. Ugly internal feuds have also sometimes broke out: a short while ago, one board director was stabbed to death in a factional squabble over university management. Most of the scandals have been restricted to the smaller schools but even the leading universities have not been immune; one of the bigger medical schools is at present under fire for bending entry requirements.

Now, the Federation of Private University Associations, to which all but 4 per cent of the private universities belong, is preparing guidelines for the public voluntary announcement of financial reports. It is hoped that greater openness will give the universities the public support they need to halt the steady cuts in the government subsidies.

In the 1982 fiscal year, subsidies were frozen, in 1983 they were cut by 2.3 per cent and in 1984 by another 12 per cent. Less than 20 per cent of universities' expenses are now met by the government; a few years ago it was 30 per cent and the original plan was to finance 50 per cent of their expenses.

The result is that college deficits have hit new highs. The 82 members of the Japan League of Private Universities, including such important universities as Keio and Waseda in Tokyo and Dooshisha in Kyoto, have run up cumulative deficits that passed the 100,000 million yen (US\$416 million) mark at the end of the last fiscal year. With income not covering expenditure in 45 universities, cuts are being made in facilities and scholarships.

The government is already considering another cut next year, or at best a one per cent increase which will not even match inflation. There is little doubt that government officials, faced with Finance Ministry demands for further austerities, have taken advantage of criticisms of high salary levels in the private universities to single them out for cuts. But at the same time, their policy makes little sense in the light of the present

demographic situation and calls for increases in the number of researchers for Japan's "high-tech revolution".

As the children of the post-war baby boom reach university age, a massive increase in the number of university students is expected: the Ministry of Education is expecting an additional 86,000 enrolments a year in the early 1990s. But the boom will be short-lived — it will last only from 1986 to 1992, after which the population of 18-year-olds will fall dramatically. At present, 80 per cent of students go to private universities and the Ministry of Education wants to keep this constant; 80 per cent of the bulge is thus to be taken up by private universities. But far from preparing for the rush, private universities are now actually cutting back.

The only other way to pay for new facilities is to increase tuition fees, but there are signs that they are already close to what the market can bear. The average Tokyo family already spends 64,000 yen (\$267) a month on education. For those with a child

at a private university and another at a private high school, average expenditure on schooling comes close to 40 per cent of total living costs.

What seems likely in the near future is that private universities will simply take on more students without a substantial increase in staff or facilities. This will inevitably lead to a decline in standards. Private schools on average already spend per student only a quarter of what a state university can afford, the student-professor ratio is three times as high and classroom space per student one-third that in a state university. Few private universities can match state university standards.

These are problems that will clearly need urgent discussion in Prime Minister Yasuhiro Nakasone's Educational Reform Council, which has already begun its deliberations (see *Nature* 309, 739; 1984). Nakasone has himself stressed the need for increasing diversity in higher education, which he believes can be provided by the private sector. But the council has so far concerned itself largely with primary and secondary education and by the time it delivers its final report in 1986 the student boom will have begun. **Alun Anderson**

Gilbert resigns from Biogen

Washington

Dr Walter Gilbert, who gave up his faculty position at Harvard University to run the biotechnology company he founded, has now resigned as chairman of the company, Biogen. His action came after months of rumours about friction between Gilbert and members of the board, particularly those members with business experience.

Gilbert will continue to serve on Biogen's science advisory and supervisory

part of a general "house-cleaning" designed to increase efficiency and check the company's overly rapid growth. Genex, another biotechnology company that has been growing very rapidly, announced a similar retrenchment this month.

Even with the lay-offs, Biogen will have grown 20 per cent this year, and further staff increases are expected in 1985. The company has a strong financial footing, with enough amassed capital to continue current programmes for several years before marketing its first products, according to Scott King, an analyst for Montgomery Securities. Although the company was able to raise only half of the \$60 million it had sought for a limited research and development partnership, it was able to put up the remaining \$30 million from internal funds.

Zsolt Harsanyi, vice-president of E. F. Hutton, says that some of the company's rapid growth may be attributable to the phenomenon, familiar on Wall Street, of "presentation of growth" — the desire of a company going public to give the appearance of rapid expansion.

The explanation may apply to Genex as well, although Genex seems to have run into more serious financial straits. A Genex spokesperson said that a number of costly design changes had been necessary in its phenylalanine plant, at present under construction in Paducah, Kentucky. Phenylalanine is the starting material for aspartame, the soft-drink sweetener. The lay-offs are expected to save Genex more than \$1 million a year. **Stephen Budiansky**



Dr Walter Gilbert with new chief executive Mark Skaletsky

boards. A new chairman is expected to be chosen in February.

Biogen recently fired 16 per cent of its staff, a major cutback that some industry analysts interpreted at the time as a message to Gilbert of the board's dissatisfaction. Spokesmen for the company maintain, however, that the lay-offs were

Proton decay

Italy's largest hole in ground

ITALIAN high-energy physics is now the proud possessor of the largest artificial subterranean cavity in the world. The objective is to mount a refined measurement of proton decay. But how this will be done will be decided only after an international meeting to be held in Rome next April.

The 100,000-m³ cavern, 4,000 metres under the Gran Sasso in the Apennines, is a by-product of the construction of what is claimed to be the largest pair of road tunnels in the world, linking Rome by autostrada to the Adriatic coast. The cavern has cost the Italian physics budget nothing, but has been built at a cost of 80,000 million lira (£32 million) by the Ministry of Public Works, partly as a means of providing jobs. But now it must be equipped, at a cost estimated at 80,000–100,000 lira.

The Gran Sasso experiment could become the most advanced proton decay measurement in the world, if only the money for instrumentation is forthcoming. According to one British physicist involved in another proton lifetime experiment, the Italian plans are "very futuristic" and the physicists may be "slightly embarrassed by the size of their hole". Proton decay is one of the testable predictions of "grand unified theories" linking all fundamental forces except gravity in a single theory; the existence of magnetic monopoles is one of many others.

Professor Antonino Zichichi, the ex-president of the Italian institute for nuclear physics (INFN), who planned the Gran Sasso hall, says that while some of the cost will be met from Italian sources, international support from Europe and the United States will also be necessary. Next April's meeting will debate the nature of the equipment to be installed in Gran Sasso, and specifically whether it should be 10,000 tons of water (to detect decay products such as pions, which travel faster than the speed of light in the medium, by their Cerenkov radiation), or some other form of particle detector.

Water is the cheapest solution, but another 10,000-ton water experiment has been under way in a salt-mine under Lake Michigan for a year without detecting any definite decays. The simplest grand unified theory is now generally considered ruled out by the Michigan result. So it may be necessary to equip Gran Sasso to detect, for example, slow kaons (too slow to make Cerenkov light in water) which result from proton decay in the more sophisticated grand unified theories now favoured.

Other proton decay experiments under way or planned in this area are:

- An Italian measurement 5,000 metres under Mont Blanc, which has been running since 1981 but with a small (150 ton) detector mass. After the Michigan result, it

now seems that the detectable lifetime of the proton will be beyond reach of this experiment, although it has recorded one unexplained event.

- An experiment in the Kolar gold mine in India, of similar scale and age, which claims three unexplained events.

- A Franco-German experiment in the Fréjus rail tunnel 4,900 metres under the Alps, between Turin and Lyon, using 1,000–1,500 tons of water crossed by thousands of detector-sandwiches. Although this experiment uses water, it may be able to detect kaons through decay products of the kaons themselves.

- A 1,000-ton calorimetric US-British

experiment that is under construction 1,800 metres deep in an iron mine in Minnesota.

Dr Wade Allison of the University of Oxford, who helped design the detector, says this Minnesota equipment will be well able to detect kaon decay modes, and that it may not be necessary to build detectors as big as 10,000 tons to get interesting results. All existing detectors are finding "inexplicable" events at the rate of 10 per year per 1,000 tons of detector, he says. These events are isolated in the chamber and do not, on the face of it, seem to be proton decays. But he suggests that they may be strange interactions of neutrinos emanating from cosmic-ray showers in the upper atmosphere or even experimental artefacts, but that the Minnesota experiment should decide. **Robert Walgate**

High-energy physics

Italian budget to be doubled

THE Italian institute for nuclear research (INFN), which supports most of Italy's basic research in high- and low-energy nuclear physics, is likely to have its budget for 1985 more than doubled, to a total of 194,400 million lira (£85 million). This sum excludes the 50,000-million lira subscription to CERN, the international organization for nuclear research near Geneva.

This increase is a mark of the Italian government's will to build on Italy's important position in world high-energy physics, attested by Carlo Rubbia's recent Nobel Prize for the discovery of the W and Z particles. The increase will pay for Italian participation in HERA, the German electron-proton collider now under construction in Hamburg, a large proton lifetime experiment under the Apennines and other major projects including a study of a 50-TeV proton-antiproton collider (the ELOISATRON) which might be built in southern Italy.

This expansion in Italian high-energy physics makes a telling contrast with the falling support for nuclear and high-energy physics in countries such as Britain, where domestic spending has fallen by 55 per cent over the past decade. Moreover, the Italians have a lesson for the British; the problem lies with the physicists, who have failed to make their subject popular, and not with the politicians, says the now ex-president of INFN, Professor Antonino Zichichi. After "tremendous efforts" to popularize particle physics in print, in person, in theatres all around the country and on television, in flamboyant exercises which have sometimes brought him scorn among the less adventurous, Zichichi has fired the Italian imagination and won support for the first three years of his ambitious five-year plan for INFN, proposed more than a year ago. "You must convince the people first", says Zichichi, "and the politicians will follow".

This is why, Zichichi claims, the Italian parliament's budget committee and the lower house have already approved his plan. The senate is expected to follow soon. The figures give INFN 194,400 million lira next year, 220,000 million in 1986 and 230,000 million in 1987, even above the rate implied by the 1 million million total requested in the five-year plan.

INFN's present president, theorist Nicola Cabibbo, is cautiously awaiting the



senate's decision before making any statement, but Zichichi considers the senate vote a formality.

Apart from rousing popular support for high-energy physics, Zichichi also claims to have shown politicians the benefit of industrial involvement. Italian companies will be building many of the superconducting magnets and some of the electronics for the German HERA, thus putting Italy in a good position in a field where the technology is changing rapidly and industrial applications cannot be far off. The proton lifetime and other experiments in an artificial cavern under the mountain Gran Sasso has also provided employment in a region where jobs are scarce. During his campaign for a bigger budget, Zichichi has zealously exploited all these side-effects of high-energy physics. To those who have laughed at him, it seems he can now laugh back. **Robert Walgate**

Genetic pesticides

Monsanto goes ahead with trials

Washington

THE Monsanto Company will shortly notify the Environmental Protection Agency of plans for the first field test of a genetically-engineered microbial pesticide. In doing so, Monsanto will become the first company to break with the convention whereby private companies have voluntarily sought approval for genetic engineering experiments from the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH).

The proposed test consists of planting corn seeds coated with a modified strain of *Pseudomonas fluorescens* expressing the gene for *Bacillus thuringiensis* toxin. This toxin has been used for many years as an insecticide, and protects growing corn plants from attack by black cut worm. *P. fluorescens* is found naturally in association with corn roots, and it is hoped that the modified bacterium could be a continuous *in situ* source of insecticide.

The company's decision to sidestep RAC was doubtless influenced by a preliminary injunction granted last spring by US District Court Judge John Sirica which prevents NIH from approving field trials of recombinant organisms without first preparing a formal environmental assessment on a field trial "program". The case is now before the US Court of Appeals. However, the injunction applies only to institutions that receive federal funds for research. Since Sirica's injunction was granted, the Environmental Protection Agency (EPA)

has moved to assert its jurisdiction in this area. The agency published an interim policy for field trials of modified organisms in October (see *Nature* 25 October, p.695).

Under the new EPA policy, 90 days' notice must be given for field tests of "genetically altered or manipulated" organisms. The test will be allowed to proceed if EPA raises no objection. Although EPA will not formally call on RAC to examine the experimental protocol, there is "total agreement" between EPA and RAC on the sort of data that are needed to support a notification, according to Dr Amy Rispin of EPA. EPA has representatives on some RAC subcommittees, thus ensuring "commonality of approach". Its actions in moving to regulate environmental releases of altered organisms have generally received high marks in the industry. EPA will require information on how the modified organism has been constructed, on the ecology of the host organism and on how release will be performed. Outside consultants will be employed to review areas where EPA has insufficient in-house expertise.

Monsanto has conducted preliminary greenhouse and growth-chamber experiments with *P. fluorescens* which indicate that it survives in corn plant roots for only 8-10 weeks if applied to the seed at planting. The modified strain does not even survive that long, according to Dr Robert Kaufman of Monsanto. Levels of expres-

sion of *B. thuringiensis* toxin are comparable with those in the source bacterium, and other metabolites also appear to be expressed at normal levels. *B. thuringiensis* toxin is specific to lepidopterous insects in its effects.

The test will be conducted at a research farm near Monsanto's headquarters at St Louis, Missouri, on a plot measuring less than one acre. The persistence of the modified bacterium will be monitored by tests specific to the toxin gene and the protein product. Kaufman points out that the bacterium is easily destroyed by household bleach, and that fumigation with methyl bromide could also be used to prevent the spread of the organism should it not behave in the test as expected. If the bacterium does decline after a few weeks, like the natural variety, it may be left in the soil over winter to see whether it will reappear spontaneously the following year. Kaufman will not be drawn on whether the new organism is likely to be economically competitive as a pesticide, saying the modified organism to be treated is still a "prototype".

Tim Beardsley

US food agency

Passing the hat?

Washington

THE new Commissioner of the Food and Drug Administration (FDA), Frank Young, seems to have taken to heart the spirit of the season — budget-cutting, that is — by circulating within the agency a proposal to defray some of FDA's research costs with private contributions.

The idea is apparently not a new one; each time it has come up before, worries over the obvious conflict of interest problem have resulted in a quick burial of the proposal. But given the enthusiasm of President Reagan and his appointees for "user fees" and for government-industry cooperation, the idea may survive at least a bit longer this time around. It has in fact received a strong endorsement from the scientific advisory board of the National Center for Toxicological Research, the FDA laboratory in Arkansas that carries out studies of the health effects of food additives, drugs and other substances.

As foreseen by Young, corporations and private foundations would contribute to a "blind trust" that would support the agency's research. Contributors would not be able to earmark their money for any specific projects. Presumably, the contributors from the food and drug industries would benefit in particular from research carried out by FDA on test methodologies. (In general, the manufacturers are required to perform their own safety testing on products they submit to FDA for licensing).

An FDA spokesman emphasized that Young had in no way committed himself to the idea and that, in any event, its implementation would require legislative approval.

Stephen Budiansky

Agencies vie for control

Washington

THE Environmental Protection Agency (EPA)'s competence to regulate environmental releases of genetically engineered microorganisms came under harsh scrutiny last week in Congress. At a hearing on biotechnology chaired by Representative John Dingell, EPA's assistant administrator for research and development, Bernard Goldstein, was grilled on an internal EPA memorandum asserting that the agency had "insufficient resources and no designated management plan for developing a cross-cutting biotechnology assessment programme in 1985".

The memorandum, by John Fowle, biotechnology coordinator in EPA's office of research and development, indicates that the office has 6.9 full-time equivalent staff and \$1.3 million dollars committed to biotechnology assessment in 1985. It concludes that 20 full-time staff and \$4 million would be required for a "credible" biotechnology programme, which would have 20-30 per cent of resources devoted to fundamental research.

Representative Gerry Sikorsky said that biotechnology assessment was low on

EPA's list of priorities and lamented that he "heard a lot of good talk" about regulation of biotechnology but that "there's little going on so far". Dr Goldstein said the biotechnology budget for fiscal year 1985 within the agency was more than \$1.6 million, but that the total budget for process regulation exceeded \$50 million.

EPA is not the only agency where attention is being given to regulation of biotechnology. Orville Wright, assistant secretary for science and development at the US Department of Agriculture, endorsed a proposed National Biological Impact Assessment Program to be located within the department. The programme would be research-based and would carry out "stepwise" assessments of proposed environmental releases of modified organisms. It is unclear how such a programme would be used by other regulatory agencies. Dr Bernadine Bulkley of the White House science office said the office would shortly propose a new interagency scientific review mechanism to ensure the best scientific advice is brought to regulators and to minimize overlapping jurisdictions.

Tim Beardsley

Laboratory animals

Research alarm at Israeli bill

Rehovot

ISRAELI researchers are concerned that a bill for the regulation of experiments on animals now before the Knesset will, if passed, seriously impede research.

Introduced by M.K. Eliahu Speiser, it prohibits experiments "except where they are essential". Exactly what will be considered essential is to be defined by Minister of Health Mordechai Gur after consultation with a special nine-member advisory committee, which would include two doctors, two veterinarians, a representative of the Association for the Prevention of Cruelty to Animals, the director of the Biblical Zoo, representatives of Tel Aviv University and Bar-Ilan University and, last but not least, a rabbi recommended by the Minister of Religious Affairs.

The bill calls on each research centre to appoint a person who will supervise experiments on animals and who would bear criminal responsibility — which could mean a fine of up to US\$17,000 and/or a year in gaol — if the regulations governing such experiments are violated by anyone at his institution.

Weizmann Institute Professor Benjamin Geiger, who heads the institute's committee of experimental animal users, agrees that experiments must be regulated but is not pleased with the bill as it now stands. What bothers him in particular is the make-up of the proposed advisory committee, which he believes should be composed primarily of active scientists with both a concern for the welfare of animals and an understanding of research needs. Such people would be aware of the pitfalls involved in any attempt to define "essential research". After all, Geiger notes, "probably not more than 10 per cent of all experiments yield applicable results, but if you don't carry out the whole 100 per cent you'll never reach the important 10 per cent".

Geiger also challenges those who would bar experiments on animals if the results are "only" of industrial or commercial significance. "Whatever importance one attaches to cosmetics, for example, it would be irresponsible to market new ones before they have been tested on animals", he declares.

Finally, Geiger does not think that the problem of animal mistreatment is likely to be widespread in Israel because major research centres here already abide by their own strict animal welfare regulations as well as those set down by the US National Institutes of Health and other grant-making agencies.

The head of the Weizmann Institute's "Helsinki committee" (for approving experiments involving human beings), Professor Irun Cohen, raises another question. "How is it possible", he asks, "to

have an outside body threaten scientists who use animals in their experiments with the imposition of a stiff fine and even a gaol sentence while allowing them to police themselves when it comes to studies on people?"

The Speiser bill is supported by Israel's Animal Liberation Group headed by Dr Andrew Menashe, a veterinarian with the Israel Society for the Prevention of Cruelty to Animals. In fact, Menashe thinks the bill does not go far enough, and he looks forward to the day when tissue culture tests and computer modelling will be substituted for all experiments on animals.

Like his counterparts elsewhere, Menashe also argues that tests on animals say very little about people. While noting that *in vitro* studies are adequate in some cases, in others, he argues, "you can't jump straight from Petri dishes to people".

Speiser agrees. Indeed, as an ex-biologist whose wife is a doctor, he realizes the importance of biological research, including that carried out on animals. "But", he declares, "these experiments must be properly supervised, and they aren't at present."

Nechemia Meyers

Academic open shop

Tokyo

JAPAN'S Ministry of Education is shortly to take steps to loosen up the academic system by enabling people from the non-academic world to become university professors.

At present, a strict set of ordinances, the university chartering criteria, makes it virtually impossible for anyone who has not followed a conventional academic career to become a professor or assistant professor in a state university. A professor must, for example, have either a doctorate or equivalent university research experience, or must have taught for more than a certain number of years at a university. Under the regulations now being planned, any person "recognized as competent in education and research" would be considered as eligible for a professorial post.

The change comes in response to the rapid growth in the number of highly talented researchers working outside the academic system — in research laboratories in private industry for example. Similarly, other trained people who could have something to offer the universities — judges, lawyers, ex-ambassadors and journalists, for example — will become eligible for university posts. Indeed, many such people already lecture in universities but are restricted to the status of "part-time instructor". If this injection of "new blood" proves successful, it is likely that further changes will be made to allow foreigners the same access to university posts.

Alun Anderson

New Year firs

Soviet *yolki* substitutes

CONSCIENTIOUS Soviet citizens should celebrate the coming new year without the traditional decorated fir-tree (*yolka*), the weekly *Literaturnaya Gazeta* urged at the beginning of October. A special correspondent from Armenia, Zorii Balayan, gave a glowing account of the 25-year campaign to outlaw the felling of fir-trees for *yolki* which resulted, last New Year, in not a single fir-tree being sacrificed to the festivities. The outcome, Balayan wrote, was an estimated saving to the economy of some 1.5 million trees which could now go to the paper and cellulose mills, the building industry or for the manufacture of vitamin C. Who will follow the Armenian example, asked the *Literaturnaya Gazeta*?

This is not the first time that *yolki* have been discouraged in what is now the Soviet Union. In 1914, Christmas trees were banned by the Holy Synod of the Orthodox Church as a "pro-German" influence (in spite of more than 200 years of cultural acclimatization since their formal introduction as part of Peter the Great's policy of "westernization"). They made a full come-back into Soviet life in 1941–42, when the necessities of war demanded the fostering of all national traditions, properly cleared of undesirable class and/or religious connotations.

Armenia, which is far distant from the North European traditions which engendered the mid-winter custom of decorating fir-trees, must have seemed a suitable area from which to eradicate the custom altogether. The first law forbidding the felling of *yolki* was passed in 1959, but in spite of numerous "educational" campaigns, felling of firs stopped only when every family could be provided with a reasonable plastic substitute.

Meanwhile, elsewhere in the Soviet Union, particularly in the northern and western republics, the annual felling of trees, including "poaching" from parks and nature reserves, still persists. A somewhat pathetic announcement broadcast on the Moscow home service by the Ministry of Light Industry of the Russian republic in mid-October offered conservation-minded citizens two variants of plastic trees, one with shaggy "snow"-covered branches and a new "fluffy" model, produced from granulated polythene by injection moulding. Both types had been awarded the state "quality mark", it was stressed.

How far the Soviet public has responded to this virtually unprecedented "commercial" will be seen only on New Year's Eve. It is noteworthy, however, that so far no educationalist has thought fit to revise the song Russian children sing around the tree, "All in the woods was *Yolka* born, All in the woods she grew!"

Vera Rich

European accelerators

Social scientists' attack on CERN

EUROPEAN high-energy physicists are so committed to the future of one laboratory, the European organization for nuclear research (CERN), near Geneva, that peer review is no longer a disinterested mechanism for judging the validity of CERN projects, two University of Sussex social scientists claim in a paper in the journal *Research Policy*, published today.

Rather, they claim, "systematic data" on past performance and future prospects of CERN, and laboratories like it, must be collected, and presented in a form "accessible not just to researchers in the speciality concerned, but also other scientists, policy-makers and even the general public". The authors, Ben Martin and John Irvine have, of course, done such a thing.

This paper* is the third and last of a series on CERN, and it concentrates on the decision-making that led to the adoption of LEP, the large electron-positron collider, as CERN's next project. But whereas the authors' previous papers have amounted to no more than historical research, the latest instalment strays into policy statements that are inevitably contentious.

The LEP decision was a mistake, Martin and Irvine imply, made possible by lack of objective external assessment in the peer-review system. LEP should be producing results by 1988, giving detailed data on the Z intermediate vector boson (and other phenomena such as the top quark). But a four times cheaper machine, the Stanford Linear Collider (SLC), may produce similar data a year earlier, reducing LEP's usefulness, Irvine and Martin assert. And although the LEP decision was taken before SLC became a real possibility, it could have been reversed when SLC was approved, the authors have since argued.

Martin and Irvine's earlier studies show, they say, that a clear factor determining the scientific success of an accelerator is its uniqueness. Since LEP will not now be unique, it "raises the question... whether such major decisions, which have implications for other areas of basic science... should remain solely in the hands of the high-energy physics community".

The authors also say that the US studies of the 40-TeV "superconducting super-collider" (SSC), and similar but less-advanced studies of a "large hadron collider" (LHC) in the LEP tunnel, are a sign that another round in the intercontinental high-energy "arms race" is in the offing.

CERN director-general Herwig Schopper pointed out last week that discussions to avoid duplication of accelerators are already taking place in a high-energy physics panel established at the Versailles summit in 1982, and that SSC and LHC "are not projects yet — there are still various options". Moreover, Martin and Irvine's own work shows that one of

the main arguments being used for LEP in the late 1970s was its complementarity with US machines (in particular the Fermilab Tevatron, a proton collider in Chicago).

Even the late-starter (and earlier finisher), SLC, is complementary to LEP, Schopper argues, because it is primarily a machine physics experiment (to see if a

linear accelerator could be used effectively to produce colliding beam physics, a technique needed if even higher-energy electron colliders than LEP are ever required). Also, whereas SLC is confined to producing 50-GeV beams, LEP is designed to go on up to 100 GeV. "There will be some overlap in the initial phase, but that I think is a minor point", Schopper says.

Robert Walgate

*CERN: past performance and future prospects. III. CERN and the future of world high energy physics. *Research Policy* vol 13, no. 6, pp 311-342 (1984).

Campaign for research

Alliance for Science

THE Alliance for Science, a campaign for increased British spending on research and development, was launched in London last week. It has been mounted by three trades unions claiming to represent more than 100,000 scientists and technologists working in Britain. Stan Davison, deputy general secretary of the Association of Scientific, Technical and Managerial Staffs (ASTMS), said that the alliance had grown out of general concern over a considerable length of time. Government, education and industry were all to blame for the decline, he said, and workers in all these fields were represented by the campaign's constituent organizations, the Institution of Professional Civil Servants (IPCS), the Association of University Teachers (AUT) and ASTMS.

Mr Davison argued a case for innovative investment, one of the campaign's catchphrases. The organizers believe that this is an opportune time to launch the campaign,

with bodies such as the House of Lords Select Committee on Science and Technology and the Engineering Council asking that something should be done. The campaign will continue at least until the annual conferences of the political parties next autumn. The organizers are calling for a body for research to do the job that the National Economic Development Council does for the economy.

Ms Diana Warwick, general secretary of AUT, complained last week that the British government underestimates the amount of cooperation between industry and higher education, but said a "balance within universities between basic and applied research" is needed. She said that many developments in solid-state physics and X-ray crystallography originated from academic research programmes, and boasted that the biotechnology department of the University of Wales had turned a redundant dairy into an important centre for biotechnology.

The organizers are downcast that the number of graduate students fell by 20 per cent during 1979-83 because of restrictions on the science budget. And the annual growth rate in expenditure on research and development in Britain is now a third of that in Japan and a half of the comparable figure in West Germany. Twenty years ago, Britain led the field. "It is now very clear", Ms Warwick said, "from detailed reports and the University Grants Committee report, that the cuts have fallen on research rather than teaching. The equipment grant is over £16 million less than what it needs to be to keep pace with rising costs."

Bill Brett, assistant general secretary of IPCS, forecast at the alliance launching that there will be, between the British scientific civil service and the research councils, a further cut of 20 per cent by 1988. He attacked the "new phenomenon" in the scientific civil service of short-term contract research staff. The attendant insecurity was not conducive to satisfactory achievement. "Science has had a bad reputation", he said, "it has been associated with weapons and information technology of the worst kind." IPCS is eager to have people asking whether the United Kingdom is spending enough money on research.

Hugh Barnes

GLORIA scoops pool

A NEW model of the British side-scanning sonar GLORIA is to be built by the Institute of Oceanographic Sciences (IOS) under an agreement signed this week between the Natural Environment Research Council (NERC) and the US Geological Survey. Under the terms of the six-year agreement, NERC, which will lease back the sonar from the Americans, will survey the remaining five million square miles of the US exclusive economic zone, mainly in the Pacific and around Alaska. The mapping of seafloor features that may hold significant mineral deposits is part of a policy of assessing the long-term economic potential.

The total cost of the package, including charter of the research vessel *Farnella*, will be about £12 million. Although the Mark III GLORIA will be US-owned, the arrangement has the advantage of allowing IOS to use the existing version elsewhere. Completion of the new torpedo is planned for March 1986, with surveying commencing that summer. In the interim, mapping of the Gulf of Mexico will be carried out using the existing GLORIA.

Peter Gambles

Genetic engineering

Rifkin's foot in the door

Washington

AT a hearing this month, the US Court of Appeals gave some preliminary indications of how it will rule on the motion by the National Institutes of Health (NIH) and the University of California to lift a lower court ban on field trials of recombinant organisms. Although a decision from the three-judge panel is not expected for several months, the judges last week repeatedly interrupted attorneys for NIH, the university and Jeremy Rifkin — the antigenetic-engineering activist who obtained the lower-court injunction — with questions that suggested how they would vote.

The injunction was based on a finding by US District Court Judge John Sirica last spring that NIH must conduct a formal "environmental assessment" before approving the experiment proposed by Dr Steven Lindow of the University of California, in which genetically-altered bacteria were to be sprayed on potato plants to provide protection against frost. Even though the proposal was discussed at two open meetings of NIH's Recombinant DNA Advisory Committee (RAC) and published in the *Federal Register* for public comment, Sirica found that the law requires a formal document; he also concluded that RAC and NIH failed to consider possible environmental consequences of the experiment such as the effect of the bacteria on nearby plants and insects.

The preliminary injunction granted by Sirica also blocks NIH from approving any other field trials of recombinant organisms until NIH prepare an environmental impact statement on the full programme of field trials. NIH say that such a statement would take a year to prepare, and would be of little value because of the widely disparate nature of the field trials that are likely to be proposed. NIH have agreed, however, to prepare the much briefer environmental assessments on each field trial that is proposed. The University of California is continuing to challenge both the programmatic environmental impact statement requirement and the need for an environmental assessment on the Lindow experiment.

At the hearing, Judge George MacKinnon, who is generally considered to be the most conservative of the three, appeared to accept the government's argument that the RAC guidelines require the equivalent of an environmental assessment on each proposal submitted, whether or not there is a formal document with that name on it. And he several times sharply challenged Rifkin's attorney on the need for a programmatic environmental impact statement (EIS), pointing to precedents that established such a requirement only for programmes with a clearly cumulative effect.

Judge Abner Mikva, who is regarded as a liberal, suggested that if NIH were required to prepare such a statement, it would of necessity be vague and hypothetical; he told Rifkin's attorney that if his goal was closer scrutiny of the environmental consequences of field trials, "you might be better off without a programmatic EIS — frequently an agency will issue a broad EIS and then refer to that to cover all their sins".

If what appears likely happens — that the court allows RAC to proceed with approval on a case-by-case basis, but requiring an individual environmental assessment for each — Rifkin will still have gained the foot in the door that he is after. By establishing that RAC's review proce-

dures are subject to the National Environmental Policy Act — the law that requires environmental assessments and environmental impact statements — Rifkin will open the way for routine court challenges every time RAC approves a field trial; he will presumably argue that the environmental assessment in each case was not adequate or that a full environmental impact statement is required. Rifkin has said in several interviews that there should be a "moratorium" on field trials until a "framework" for assessing all the risks is established, a condition which he maintains is not possible with current knowledge. And he has also said that any environmental assessment should be done on a "worst-case" basis.

Meanwhile, RAC's staff is proceeding with preparation of an environmental assessment of the Lindow experiment.

Stephen Budiansky

European Parliament

Commission's budget rejected

THE massive vote by the European Parliament last week to reject the whole 1985 budget of the European Commission could mean trouble for the European fifth-generation computer project, Esprit, and for other scientific projects planned to grow or start afresh next year.

Esprit, for example, which links individual European computer and electronic companies in joint pre-competitive research projects, cost 45 million European accounting units (ECU) in 1984 (some £25 million). The programme was due to more than double to 100 million ECU in 1985. But under the rules governing the operation of the European Community without an official budget, spending must go on forward by "provisional twelfths" — each month the Commission will be able to spend no more than a twelfth of total spending in the previous year, in each individual line of the 1984 budget.

Of eight major programmes tabled for discussion at this week's meeting of research ministers (see *Nature* 15 November, p. 187), five are likely to receive no cash at all in 1985 until the battle with the European Parliament is settled. The non-nuclear energy programme, for example, may come to a total halt — its second programme finished 18 months ago, and since it had no official budget line in 1984, under the rules it cannot be funded in any fashion next year.

European parliamentarians, who have largely rejected the budget in a play for greater influence over the radical restructuring of the Community now under discussion among European heads of state, were last week attempting to play down the damage their decision has done to research. But the damage can be limited: some new programmes, it is pointed out in Brussels, must go through lengthy exercises of setting up expert committees and putting

out calls for tender. If the council of ministers meeting this week approves some of the programmes in principle, it may be possible to get through a large part of 1985 without spending an ECU, while nevertheless making some useful preparations to spend when the purses are filled again.

Robert Walgate

Hunting heads

THE Department of Education and Science is taking the un-British step of asking a head-hunting firm to suggest candidates for posts as the head of two research councils due to become vacant next year. The posts concerned are those as chairman of the Science and Engineering Research Council (which Professor John Kingman will leave at the end of August 1985) and chairman of the Natural Environment Research Council (which Mr Hugh Fish will vacate at the end of September 1985).

This unusual step appears to have been forced on the government by the dearth of acceptable names reaching it from the usual nominating bodies that constitute the old-boy network. The head-hunters, John Stork and Partners, have been retained only as advisers, and will be expected to provide Sir Keith Joseph, the Secretary of State for Education and Science, with a list of names from which to make a choice.

Part of the difficulty attending this is that the salary offered, that of an under-secretary in the civil service (now just over £30,000 p.a.) is too low to attract able people from industry and hardly enough to compensate senior academic scientists for the invidious responsibility of administering policies (or budgets) that must lose them friends. The usual compensation of a knighthood is not always a sufficient recompense, and cannot be traded in for heat and light. □

Symposium volumes

SIR — Anthony Watkinson's interesting and mildly cynical view of conference proceedings (15 November, p.201) represents one side of a very complex phenomenon. David Lodge, in his novel *Small World*, has explored some of the other activities of conference delegates. Judging from the locations of most US conferences and the attendant social programmes for both delegates and spouses, little time can be spent in the scientific sessions — hence the need for publication of the proceedings.

Whether or not proceedings should always be published, they are certainly wanted. The British Library Lending Division at Boston Spa has for many years tried to collect conference material and now holds about 200,000 volumes of proceedings. The library receives more than 250,000 requests a year for conference papers. A small percentage (less than 5 per cent) of these requests, but inevitably those causing the most trouble, are for papers allegedly presented at a conference but never published. Sometimes, however, the Lending Division holds papers which are never printed in the published proceedings.

I would urge that journal editors refuse to allow authors to quote references to conference papers that are not published unless this is made clear, and that organizers of conferences indicate in advance their publication policy. One way of tracing conferences, especially if the full title is not known for sure, is to consult the *Index of Conference Proceedings* published by the British Library Lending Division — a monthly keyword index cumulation, annually and quinquennially.

GARTH FRANKLAND

*British Library Lending Division,
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SIR — Anthony Watkinson's article (15 November, p.201) on the publication of symposium volumes and conference proceedings explained why publishers like this kind of book. But he only outlined in general terms why many scientists are becoming increasingly hostile to such publications.

At a recent international meeting in the earth sciences, I was approached by no less than five publishers to produce a special issue or book of the symposium that I was convening. It became clear that the publishers were not particularly interested in the academic arguments for publishing this symposium (which happened to be of only average quality), but were largely concerned with printing paper and selling it at high prices to libraries.

The most important detrimental effect of the proliferation of symposium books may in fact be on journals. The best scientific journals are required both by commercial considerations and by duty to their readership to publish at regular

intervals. They therefore require a steady flow of high quality manuscripts reporting interesting and original scientific results. A healthy situation for the progress of science in general occurs when such journals have a sufficient supply of papers to be selective and to demand high standards, yet can publish a paper within a reasonable period of time. At present, the amount of high-quality research being carried out has reached a plateau and may even be declining in some fields. The symposium books, however, not only publish mediocre papers as implied in Watkinson's article, but also publish some first-class material. Such papers are removed from the reservoir of potential material available to the regular journals. In addition, they are not always reviewed very carefully — a process that is important even for good papers. These effects may well force some journals to lower their standards since they are usually obliged to publish a certain volume of material anyway.

There is no doubt that collections of papers on carefully selected topics can be outstanding contributions to the dissemination of knowledge. I believe, however, that scientists should be much more critical about agreeing to edit and organize symposium volumes. It is clear that most publishers will not agonize much about the various advantages and disadvantages as long as there is a profit to be made. Scientists are the only ones who will have a major effect by saying no more often.

R.S.J. SPARKS

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Science and miracles

SIR — In response to your leading article on miracles (*Nature* 310, 171; 1984), I think that there are at least two different views of science.

(1) Science as a parlour game, somewhat like chess. One sets the rules, decides who may play and who not, who is the most skilful at it, and even endows some with a certain authority. In such "science" one might state that there are no flying saucers and that miracles do not occur, by simply making it against the rules. Obviously such "science" is limited in its content and truth by the rules until someone changes the rules.

(2) Science as a quest for the truth or at least a working model of the truth. We do not set the rules but try to discover them. We try to achieve some confidence in what we find by analysis, experiments and observations, and not to discolour the truth with our prejudices.

It is reasonable to believe in miracles. The Creator of the Universe might well

create a miracle, and miracles have been reported through the ages up to the present times. (Consider the happenings at Lourdes, France.) Moreover, by the criterion of fewest serial steps and most parallel steps, the existence of the Creator is one of the most certain things in science. (Demonstration is beyond the scope of this letter, but consider the statement: "I think, which shows that God is".)

(Consider in this context the statement that this *cannot* happen because it violates So-and-so's law ...". One wonders: since when does So-and-so run the Universe?).

Much of the bickering in science might perhaps be ascribed to the following of the first view of science by people, rather than realizing that we are in the same boat of ignorance, that one cannot be absolutely certain of anything that one does not absolutely control, and that one is therefore dependent on what the Creator chooses to reveal directly or indirectly.

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UNESCO and VAT

SIR — The announcement by the British Foreign Secretary that he has served notice on UNESCO of withdrawal by the United Kingdom from the organization will come as no surprise to anyone, least of all to readers of your leading article (22 November, p. 293).

International considerations apart, the move has more parochial implications for the scientific community, as it comes at a time when strong rumours persist that the Chancellor of the Exchequer has proposed the imposition of Value Added Tax on printed matter. It is not perhaps widely remembered that the proposal was considered when VAT was first instituted but rejected because it infringes the UNESCO charter. Could Britain's projected pull-out from the organization weaken resolve at home to adhere to the principles of the organization? If books, and of course journals such as your own, were so taxed, the effects on education in its widest context would outweigh all the previously inflicted damage caused by previous cuts in financial support from the teaching of literacy up to graduate level. Beyond this, the restriction of information flow that would ensue as library committees stopped journal subscriptions would be paralleled by fewer personal subscriptions and the many learned societies that rely on income from this source would be severely hit.

Were this proposal to be formalized in a finance bill it would be too late to avert its implementation. The time for objections to be voiced is surely now.

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Technical analysis of IIASA energy scenarios

from Bill Keepin & Brian Wynne

Despite the appearance of analytical rigour, IIASA's widely acclaimed global energy projections are highly unstable and based on informal guesswork. This results from inadequate peer review and quality control, raising questions about political bias in scientific analysis.

DURING the energy crisis of the early 1970s, there was urgent interest in negotiating a transition from global dependence on fossil fuels to an ultimately sustainable energy system. To analyse such questions, a large-scale research programme was initiated at the International Institute for Applied Systems Analysis (IIASA) in Laxenburg (near Vienna), Austria. In 1981, the project issued strong conclusions and policy recommendations that were widely publicized in the professional literature¹⁻⁴ and the general press^{5,6}.

While working at IIASA, we have re-examined the quantitative analysis behind these conclusions. Much of the attention given to the published study appears to have been stimulated by the sheer magnitude of the project and by its claim to be a globally comprehensive and consistent study. The general conclusion was that world energy consumption would increase dramatically over the next fifty years. This would require major expansions of all energy supply options, including fossil fuels, and a major commitment to synthetic fuels and nuclear power. For example, nuclear power was required to expand at the average rate of one new power plant (1,000 MW) every four to six days for the next 50 years.

Our work has revealed various shortcomings both in the analysis itself and in its published presentation^{7,8}. Although the overall study involved more than quantitative analysis using computer models, this scientific aspect of the study has been given prominence as a claimed means of controlling bias in an inevitably ill-structured problem domain. The relationship between formal analysis (open to external review) and essentially inaccessible informal judgements in the construction and use of policy models has become an important problem, not only in the energy field⁹. Although informal judgement based on craft experience has been recognized as necessary, its existence poses a dilemma for quality control and normal peer review. Whatever the relative influences of formal and informal processes, analysis of the models themselves can help not only to improve technical standards, but also to expose the embedded informal commitments for external debate and evaluation. Greater elaboration in formal modelling does not mean reduced scope for informal subjective judgement — indeed the opposite may be true. This explication is

especially important in the IIASA energy study because the scenarios derived from the models continue to be used as definitive benchmarks in energy policy and associated analyses (for example of the global CO₂-greenhouse problem).

The major results of the IIASA Energy Program were based on comprehensive quantitative projections, or "scenarios", of the world's energy future for the succeeding 50 years. This article focuses on the analysis behind these scenarios, which were generated from a set of detailed computer models. It is found, in many cases, that the models essentially reproduce informally prescribed input projections that pass through the models unchanged. More importantly, despite claims of robustness, the scenarios are unstable with respect to minor variations in key input data. This was recognized in early sensitivity work that was discontinued and is not cited in later documentation.

These findings bring several conclusions drawn from the scenarios into question. They also raise more general issues relating to professional standards, peer review in non-experimental science, and the proper role of science in formulating public policy.

IIASA Energy Program

The IIASA Energy Program began in the wake of the 1973 oil embargo and lasted

seven years, involving more than 225 person-years of effort and a total research budget exceeding \$6.5 million⁷. More than 140 scientists from 20 countries participated in the study, and a broad network of collaborating institutions was established (including the UN Environmental Programme, International Atomic Energy Agency, Siberian Power Institute, and the US Electric Power Research Institute, National Center for Atmospheric Research and Stanford Research Institute). The final report was published in two volumes¹⁰ in 1981, entitled *Energy in a Finite World* (hereafter abbreviated as EIFW); an *Executive Summary* was more widely distributed¹¹. The IIASA programme is generally regarded as "the most ambitious such study carried out thus far"¹², and has had a considerable impact in scientific circles¹³⁻¹⁵ and among policy makers^{16,17}. The scenarios are routinely cited as prominent benchmark forecasts^{18,19}, and have served as a starting point for various subsequent investigations^{19,21}.

The scenarios were central to the IIASA programme, and outweighed many other aspects of lasting value. Important results were produced from painstaking studies of global resource bases. Other valuable contributions include the logistic substitution model, and the IIASA work on CO₂, technological risk perception, and solar

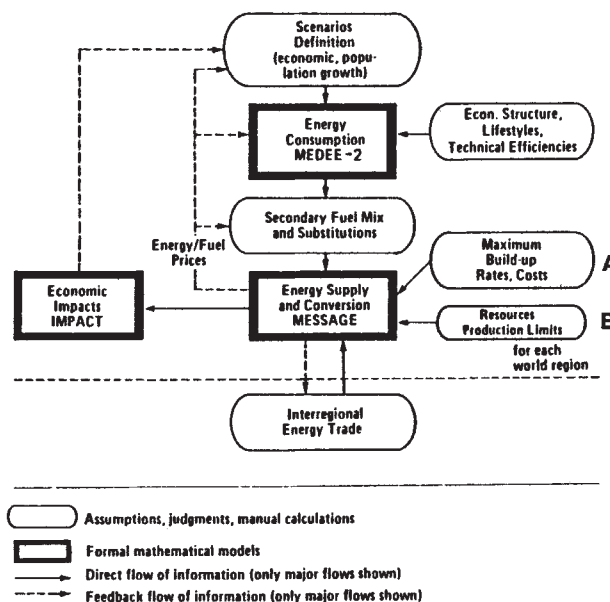


Fig. 1 The IIASA Energy Models (reproduced from ref. 10, p.401). Note distinction between formal models and "assumptions, judgements, manual calculations". Input data taken from ovals A and B are used to construct the "scenariettes" in Figs 2 and 3.

energy^{7,10}. In addition, IIASA provided an unusual forum for conferences and intercultural professional discourse. This article is therefore not a general critique of the entire IIASA Energy Program. Most participating scientists were not directly involved with the shortcomings reported here.

Our findings can only be summarized here (see refs 7 and 8 for details). This article focuses on methodology, and although there are policy implications, it neither endorses nor discourages any particular energy policy. Quotations are used extensively below to present the IIASA analysts' work in their own words. Unreferenced quotations refer to the *Science* article¹, and page numbers refer to EIFW, Vol. 2¹⁰.

IIASA energy scenarios

The stated purpose of the IIASA study was "to understand the factual basis of the energy problem, that is, to identify the facts and conditions for any energy policy". In this endeavour, "an explicit attempt was made to incorporate as many views and to be as objective as possible". To analyse the uncertain future, two scenarios ("high" and "low") were "constructed as a means of spanning the conceivable evolutions of global energy systems over the next 50 years" (p.656).

For this purpose, the world was divided into seven geopolitical regions¹, and individual scenarios were developed for each region. These were then aggregated to yield global scenarios, which were not intended to be forecasts, but rather realistic and pragmatic projections that "span a sufficiently wide range in order to incorporate the unavoidable uncertainties" (p.425). Conclusions were drawn from these to "provide decision- and policymakers with the information they need to make strategic choices" (p.800).

To ensure internal consistency in the globally comprehensive analysis, IIASA developed a set of mathematical models¹ which were the principal tool used in building the scenarios¹¹. These were of key importance in the analysis; according to EIFW, "the assumptions and results [from the energy supply model] represent, in some ways, the core of the energy studies reported in this book" (p.402).

A schematic diagram of the IIASA model set is reproduced in Fig. 1. Note the distinction between formal models and informal procedures. Also observe that the major flows of information circulate in a clockwise fashion, linking the formal models together into an iterative loop. This is important because the models are extremely detailed, containing several thousand variables and parameters representing different facets of the energy system. These various quantities are reportedly controlled and adjusted by repeated iteration until a self-consistent scenario is obtained.

The iterative procedure begins with

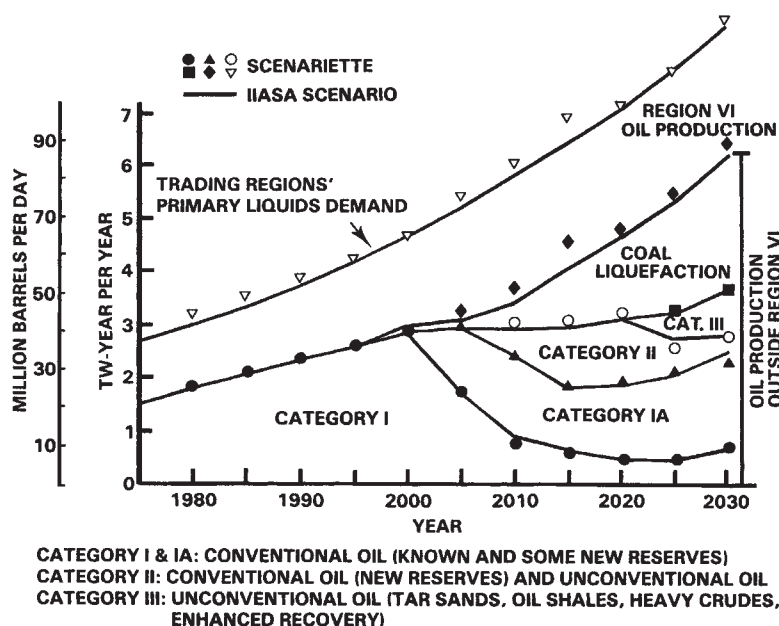


Fig. 2 World oil supply and demand projections (excluding centrally planned economies). Data points ("scenariette") are derived directly from inputs to IIASA energy models (Fig. 1). Curves (IIASA scenarios) are outputs from models. Close agreement reveals superfluous role of models. Compare Fig. 5 in ref 1.

initial assumptions about economic growth, which are combined with population projections and lifestyle parameters (in MEDEE-2) to produce projections of energy consumption from 1980 to 2030. These are fed into an optimization model (MESSAGE) which computes the least expensive supply strategy that meets the specified consumption levels, subject to constraints on resources and technology. This strategy is then fed into an economic model (IMPACT) that calculates requirements for capital investment, labour, land, water, materials, equipment and additional energy. Finally, "the main model loop is closed with IMPACT"²², whose outputs are fed back (via "the built-in feedback mechanism"²³) to modify the original economic growth assumptions. This completes one iteration cycle. Most feedbacks were manual, leaving room for an element of judgement, but the "flow of information is mechanized" (p.400), and the procedure is currently being streamlined²⁴.

In brief "the global high and low scenarios are the results of applying the model loop iteratively until satisfactory consistency was achieved"²², which in turn "required several iterations of the model set"¹¹. Note that the term "scenario", as used here, refers to the final quantitative results of a comprehensive mathematical analysis.

Role of the models

Despite sincere efforts to develop and implement the iterative procedure just described, it was never achieved. Major difficulties were encountered with the model IMPACT, which was finally "applied reluctantly and only at a place where no...

further modelling steps would be drawn from it"²⁴. Recently, it has been acknowledged that IMPACT served as a "monitoring model"^{7,8}, meaning that the iteration involved only the sub-loop between MEDEE-2 and MESSAGE, which had little effect. Indeed, only two examples of iteration are described in EIFW (pp.404-07), both involving minor adjustments in the heating sector (yielding 10% to 15% changes by 2030). Crucial variables and constraints (such as costs, build-up rates, resource extraction limits) did not enter the formal iterations. Moreover, closed-loop iteration of the three model loop was not performed, contrary to several statements in the documentation. Thus, macroeconomic constraints were not explicitly incorporated into the scenarios, and internal consistency was not quantitatively assured (a fourth model (MACRO) analysing macroeconomic effects (such as capital/output ratios) was said to be implemented earlier, but was later dropped⁸).

Given the lack of significant iteration in the model set, it is natural to ask what role the models played in calculating the scenarios. One approach to this question is to consider how the models compute scenarios from input data. For this purpose, direct comparisons of the inputs to models alongside their outputs may offer insight into the internal dynamic and analytic workings of the models themselves.

Such an approach has recently been carried out⁷. The procedure involved manual construction of a highly simplistic scenario, using only the model input data. This scenario is dubbed the "scenariette"

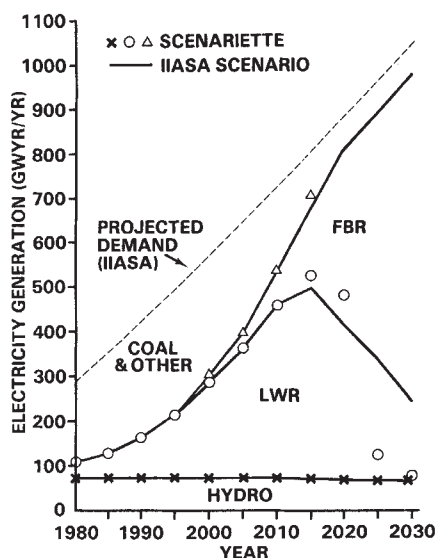


Fig. 3 Hydroelectric and nuclear power projections (for Western Europe, Japan, Australia and New Zealand). Points are derived from inputs to IIASA models (Fig. 1); curves are outputs. Rapid displacement of LWRs by FBRs is due to 2% cost difference (see Fig. 4).

to reflect the crudity of the analysis: usually it amounted simply to plotting a few sets of input data on the same graph. In some cases, it involved heuristic optimization and some minor accounting calculations (using a hand calculator), but no significant analysis (such as dynamic simulation, iteration and equation solving). Nevertheless, in many cases this back-of-the-envelope analysis reproduced the IIASA scenarios precisely, implying that the formal models are largely superfluous.

As an example, consider one of the most crucial aspects of the global energy system — the supply and demand for oil. A 50-year scenariette is constructed from time series input data taken from ovals A and B in Fig. 1. These data, which are external to the models and feedback loops, include prescribed constraint trajectories for rates of oil extraction and coal liquefaction in each region from 1980 to 2030. These are aggregated and plotted as points in Fig. 2. For direct comparison, the corresponding IIASA scenario is plotted on the same graph as a set of curves. Observe the striking agreement between scenario and scenariette. The differences between them are insignificant because precise numerical results "... must not be taken too literally. In fact, they must be seen as quantitative expressions of the more qualitative situations that underlie the scenarios".

Similar close agreements have been found in many other cases between the IIASA scenarios (both high and low) and the corresponding scenariettes⁷, including frequent identity (especially during the first 30 years). For example, Fig. 3 represents the results for hydroelectric power, light water reactors (LWRs) and fast breeder reactors

(FBRs) in a particular region (the models' only effect is to smooth the rapid phase-out of LWRs after 2015).

Although these findings raise questions about the models (discussed briefly below), the most important observation is that many of the principal scenario results are essentially prescribed by the input data — outside of the set of mathematical models. It is therefore natural to ask where these data come from, particularly since many of them are specific numerical projections extending to the year 2030. In EIFW (pp. 528–31), these data are described as "guess-timates", "rough average (sometimes consensus) estimates", "at best a zero-order approximation", or just simply "assumptions". Thus in most cases little empirical or theoretical evidence is supplied to substantiate the input assumptions, and few (if any) quantitative details are provided to indicate how the numbers were obtained. Thus, for coal extraction: "These constraints represent the roughly assessed composite and aggregate limitations of water, manpower, transport, environmental safeguarding, and so forth in each region" (p. 571). No details are given to show how these projected ceilings (on annual coal extraction) from 1980 to 2030 were obtained. For oil extraction, an outline is given (pp. 534–46).

A variant of Fig. 2 has been widely published^{1,4,10,11} as evidence for (1) the urgent need to develop synthetic liquid fuels (coal liquefaction), and (2) the necessity to exploit shale oil and tar sands, despite the tremendous environmental consequences that were not explicitly taken into account⁷. These conclusions are based primarily on the scenarios, which are now seen to be largely copies of input assumptions, which are in turn acknowledged to be informal guesswork. Thus, the analytical basis for these findings is inaccessible subjective judgement rather than formal calculation.

Sensitivity analysis

Modelling the global energy system is a formidable task, fraught with uncertainties that are greatly amplified when looking 50 years ahead. To complicate matters, long-term projections cannot be verified, and the time span of available data is too short to apply standard model validation tech-

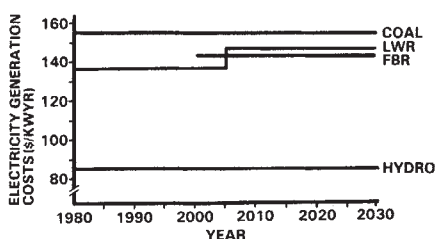


Fig. 4 Future costs of electricity generation assumed in IIASA scenarios. A small increase for LWRs in 2005 yields 2 per cent advantage for FBRs, producing major transitions to the breeder reactor (Figs 3 and 5a).

niques^{25,26}. In this predicament, an indispensable tool is sensitivity analysis, which explores how projected results change in response to perturbations in input assumptions. This can help to identify major trends, key policy variables, and vulnerabilities, which is a principal motivation for modelling (rather than attempting to forecast). Moreover, careful sensitivity testing is essential when formulating robust conclusions from an analysis.

The importance of this was recognized for the IIASA scenarios, and it has been asserted that "the sensitivity analysis was done — at length"⁷. EIFW devotes a full chapter to alternative scenarios and sensitivity analyses, concluding that "Together, the scenarios, the alternative cases, and the sensitivity analyses should build a broad enough understanding... so that conclusions and recommendations for the energy transition [to a sustainable future] can be formulated" (p. 614). A number of recommendations and conclusions that are claimed to be robust have subsequently been broadly disseminated.

Since the 1973 oil crisis, it has been clear that future energy costs are extremely uncertain. Consequently, the IIASA analysis "did not start on the side of costs and prices". In fact, "the desire for data robustness dictated our decision to avoid an approach relying completely on prices" (p. 27).

"All of this leads to a belief (or hope) that the scenarios here are robust, that they can stand up against events whose impacts, in human terms, may be large" (p. 395). However, our analysis shows that the scenarios are highly sensitive to minor variations in assumed costs and other parameters. Summarized below are results from detailed sensitivity tests carried out for the low scenario (for the core model MESSAGE in particular⁷). Note that this scenario is now thought to be more likely²⁷.

Robustness of scenarios

Consider the assumed evolution of electricity generation costs (Fig. 4), most of which are held constant (in real terms) throughout the entire 50-year period. Although these fixed cost projections are highly unlikely, they would be acceptable for modelling purposes if the final results were insensitive to them. However, the exogenous projections shown in Fig. 4 determine much of the model behaviour.

For example, observe that the cost curve for LWRs undergoes a small step increase in the year 2005 due to an assumed increase in the cost of uranium. The model contains two categories of uranium: cheap and expensive. When uranium in the cheap category is exhausted, the model switches to the expensive one, raising the cost of LWRs by 7 per cent, as indicated by the "LWR step".

Note further that the FBR becomes available in the year 2000. Initially it is more expensive than the LWR, but then the LWR step occurs, leaving the FBR with a

slight cost advantage (2.1 per cent). The result is that FBRs are built up as rapidly as possible, and LWRs are phased out (Fig. 3). This artificial feature of the model is the major factor determining the role of FBRs in all scenarios⁷. The electricity cost assumptions are the same in all cases, the only differences being in the temporal location of the LWR-step, which is governed by the quantity of cheap uranium available — a highly uncertain input parameter. An increase of a few per cent in the proportion of uranium allocated to the model's cheaper cost category delays the introduction of FBR by more than 30 years in some cases⁷. The IIASA scenarios were taken by many as strong evidence for the inevitable large-scale introduction of the breeder reactor¹⁵.

Other sensitivities are even more critical. Consider IIASA's 50-year projection for electricity generation in the United States and Canada (Fig. 5a). A 5 per cent increase in the assumed cost for the FBR eliminates its small economic advantage (Fig. 4), which causes the entire FBR contribution (Fig. 5a) to be replaced by additional LWR and coal capacity⁷. In another example, a 16 per cent increase in the assumed costs for nuclear power causes a shift to the radically altered projection shown in Fig. 5b (this case requires moderate increases in assumed coal extraction constraints after 2025⁷). Thus a mostly nuclear future is replaced by a mostly coal future (notwithstanding acid rain and CO₂) as a result of minor changes in input data whose values are known to be highly uncertain.

Slightly altered assumptions are thus seen to produce very different outcomes. This instability is intrinsic to the analytic structure of the supply scenarios, which means that robust future trends cannot be inferred from them. Nevertheless, several specific projections have been singled out and emphasized as robust conclusions^{27,28}. For example, "by 2030, nuclear power (LWRs and FBRs) has a total of 8.09 TWyr/yr for the high scenario and 5.17 TWyr/yr for the low scenario. Its relative share is close to 23 per cent in either case"²⁸. This requires that nuclear power stations be built at the average rate of one plant (1,000 MW) every 4 to 6 days for the next 50 years⁷. As seen above, this result is based on fixed relative cost assumptions that are not only highly fragile, but also are presumed to hold until 2030. Despite extreme sensitivity to tiny fluctuations in these assumptions (which have already occurred), this result is reported as a robust observation derived from the IIASA scenarios²⁸.

It is therefore of interest to review IIASA's sensitivity work. Three alternative scenarios were developed (nuclear moratorium, enhanced nuclear, and reduced demand), but these were not used as a basis for formulating robust conclusions and policy recommendations⁷. Rather, the high and low scenarios were used for this purpose, so it is particularly important to explore sensitivity in these cases.

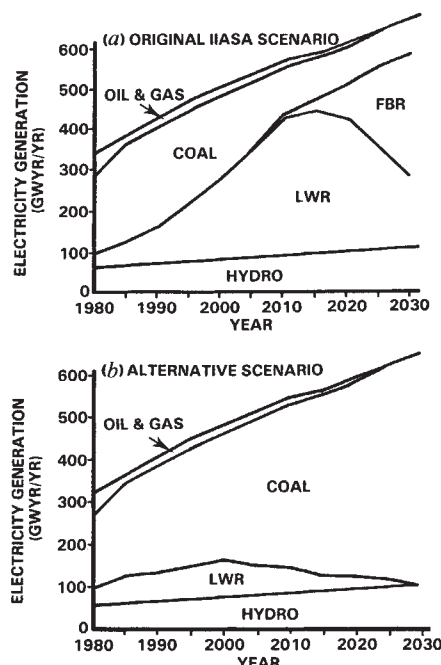


Fig. 5 Sensitivity to IIASA cost assumptions (Fig. 4) in United States and Canada, demonstrating structural instability in IIASA scenarios (which invalidates "robust conclusions" drawn from them). *a*, Original IIASA projection for electricity generation. *b*, Alternative projection, assuming 16 per cent increase in nuclear costs (and moderate increase in assumed coal extraction constraint after 2025).

After a general sensitivity discussion, EIFW treats "the more constructive consideration... of the possible variations among the relative price changes of new sources of energy" (p. 619). However, just three quantitative sensitivity tests are presented, none of which alters resource estimates or the critical relative cost ranking. The test for electricity generation assumes that all costs are doubled, thus shifting the curves in Fig. 4 upward, but preserving their relative positions. Hence the crucial sensitivities remain unexplored, and no further analyses are presented or cited⁷.

It might be supposed that the sensitivity problems we discovered are a realization of hindsight, but in fact they were recognized early in the IIASA Energy Program itself. Results similar to those shown in Fig. 5 were presented for a prototype model of MESSAGE in IIASA research memoranda published in 1974 and 1975²⁹⁻³¹. Despite the conclusion that "more work is needed in several directions"²⁹, these early findings are not cited or investigated further in the later reports and documentation⁷.

Technical discussion

Many of the technical shortcomings discussed above can be traced to characteristics of linear programming (LP) that are well known³². The single-objective LP model MESSAGE^{22,23} dominates the

IIASA analytical structure, largely because the full iterative loop was never effected. MESSAGE minimizes the total dollar cost of meeting a specified energy demand, subject to various exogenous constraints. The set of points that satisfy all the constraints is called the feasible region (or loosely, the set of all possible energy futures). A geometric representation of the LP concept is given in Fig. 6, illustrating the inherent stability problems. Given the large uncertainties in future costs of energy resources and technologies, cost minimization LP models are inappropriate for long-term robust supply projections^{7,32}. Efforts to overcome these difficulties are continuing (G.B. Dantzig, personal communication).

In many of the IIASA scenarios, the assumed constraints yield extremely small feasible regions — effectively single points. In these cases, the model behaves much like an identity transformation from the inputs (constraints) to the outputs (scenarios), as observed in Figs 2 and 3. The constraints are themselves arbitrary projections, subject to great uncertainty (to establish robustness would therefore require further sensitivity analysis — not provided in EIFW). Variations in the constraints can cause the feasible region to expand, thus magnifying intrinsic cost sensitivities (Fig. 6), to shift position or to disappear altogether, in which case the scenario ceases to exist. This last possibility may explain why the models' built-in environmental constraints, "although available, were not directly used" in the scenarios (p. 415). Since environmental constraints reduce the size of feasible regions²³, their inclusion could have eliminated the smaller feasible regions entirely, rendering the IIASA scenarios infeasible.

Certain caveats in EIFW hint at the provisional nature of the scenarios and conclusions, but these are not emphasized in articles and speeches. Recently, the minor bookkeeping role of the models in the subjective iteration process was acknowledged (Fig. 7). This figure was first published in 1983, two years after the publication of EIFW³³.

Conclusions

Two analytic results are reported here about the IIASA global energy scenarios. First, many crucial components of the scenarios are generated informally and supplied as inputs to the formal computer models, which then reproduce these projections with only minor alterations. Thus the models have not analytically "discovered" feasible energy futures. Indeed, despite the appearance of analytic sophistication and rigour, the models serve primarily as a static accounting framework for displaying the assumptions of the analysts.

Second, the supply scenarios are strongly dependent on assumed cost and resource estimates (for example, the projected expansion of the fast breeder reactor

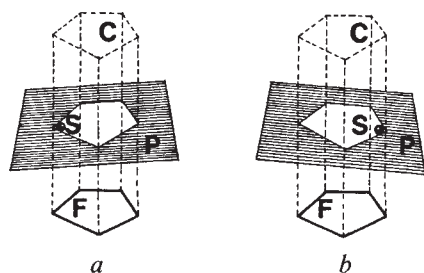


Fig. 6 Geometric representation of linear programming. *a*, Feasible region (F) is interior of polygon. Above F lies plane (P) representing total cost. Boundaries of F are projected vertically upwards (through P), defining constraint column (C). Small ball (S) is released on P inside C, and rolls to lowest point, defining optimal (least-cost) solution. *b*, Slight changes in cost assumptions tilt plane P in different direction, shifting solution from one extreme to another. This effect is observed in Fig. 5.

is due to a 2.1 per cent cost advantage introduced after 2005). Small changes in these highly uncertain estimates produce radically altered supply strategies. This inherently brittle structure precludes the possibility of drawing robust conclusions from the supply projections. Nevertheless, several conclusions have been drawn and widely publicized as robust findings, including massive expansions of fossil fuels and nuclear power.

In addition to these analytic results, various problems are found in the documentation. Early work revealing serious structural instability is not cited, and standard sensitivity tests are not included. The study's own publications have led outside scientists to believe that the formal models involved dynamic analysis and iteration for control of assumptions and genuine validation of the scenarios, rather than limited arithmetic checking. Only recently has the minor bookkeeping role of the models been clarified.

The IASA energy study claimed to reveal a narrow window of feasible energy futures (for example, "Our scenarios are globally comprehensive and allow for no escape", p.785, original italics). Now, just three years later, a broad spectrum of possible energy futures is widely recognized^{25,34,35}. However, the IASA conclusion did not result from an objective examination of possible future alternatives, but rather from embedded institutional and sociopolitical assumptions that pervaded the analysis^{8,9} (for example, expansion of capital-intensive supply technologies was emphasized, while options for reducing energy demand were largely ignored). Although the study claimed to focus only on factual aspects rather than social and political dimensions, this artificial division confined the resulting scenarios to a given institutional framework, thereby precluding full exploration of the range of possible futures. Scenarios

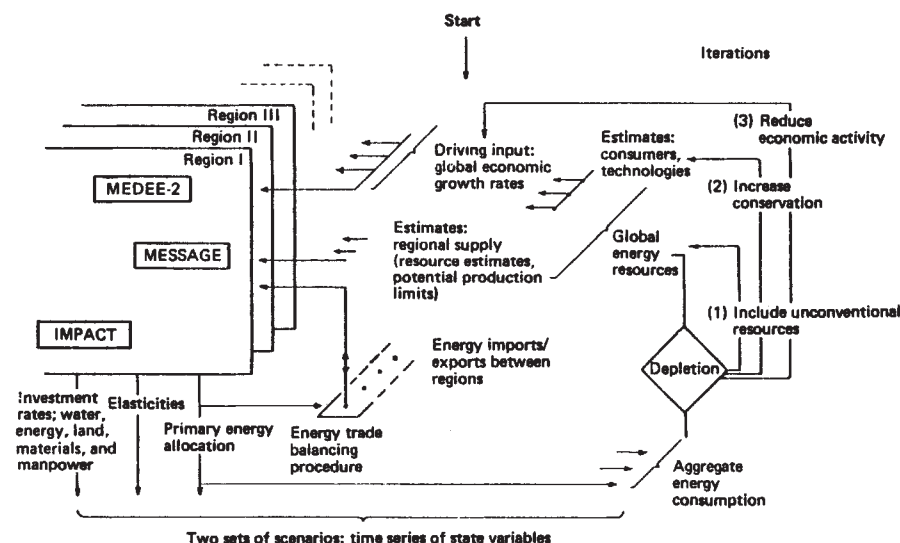


Fig. 7 New representation of scenario writing process³³, first published in 1983. Note minor role of models.

are rarely presented as forecasts, but they are frequently treated as such, even by their authors³⁶. The danger is that they will be misconstrued as inevitable, and used to justify policy decisions³⁴.

Much work of lasting value has been produced at IASA, from both inside and outside the Energy Program. These important contributions have unfortunately been overshadowed by the energy scenarios and associated urgent policy recommendations. (For example, "what must be emphasized is the advent of...synliquids and the fast breeder reactor...the most precious resource is time".) Both the urgency and specific recommendations were evidently based on opinion rather than objective robust analysis, as claimed. One important lesson is that the most exquisite formal analytic modelling still embodies informal assumptions (often about sociopolitical values and insti-

tutional behaviour) that affect what technical outcomes are conceivable. Peer review of formal models can expose these assumptions for external debate and evaluation⁷. Indeed, rather than attempting to identify objective policy truths, perhaps a more realistic role for policy modelling is to explore origins and consequences of different social and institutional assumptions^{35,37,38}. Such an approach would embrace (rather than deny) the interpenetration of science and politics in policy analysis.

We thank IASA for supporting this work, and the many individuals who helped us, especially Valerie Jones and Mike Thompson. □

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- Häfele, W. *Science* **209**, 174-182 (1980).
- Häfele, W. *Futures* **12**, 18 (1980).
- Nature **290**, 349-350 (1981).
- Sassin, W. *Sci. Am.* **243**(3), 107-117 (1980).
- New York Times, Editorial, 25 March, 1981, p.A26.
- Christian Science Monitor, 11 March, 1981.
- Keepin, B. *Policy Sciences* **17** 199-275 (1984).
- Wynne, B. *Policy Sciences* **17** 277-320 (1984).
- von Hippel, F. *Physics Today* July, 34-41 (1981).
- Energy Systems Program (IIASA), *Energy in a Finite World*, Vols 1 & 2 (Ballinger, Cambridge, Massachusetts, 1981).
- McDonald, A. *Energy in a Finite World: Executive Summary*, IASA Executive Report ER-81-4 (1981).
- Perry, A.M. in *Carbon Dioxide Review*, 1982, (ed. Clark, W.C.) 343-346 (Oxford University Press, New York, 1982).
- Phys. Technol. **12** 228 and 270 (1981).
- Energy World **83**, July, 3 (1981).
- Nucl. Energy **20**, 276-277 (1981).
- Häfele, W. *IIASA Rep.* **3**, 49-68 (1981).
- Brown, G.E. *Interfaces* **12**, 119-125 (1982).
- Ausubel, J. & Nordhaus, W.D. in *Changing Climate*, 153-191 (US National Academy of Sciences, 1983).
- Said, A. & Pochman, W. *OPEC Review*, Vol. VIII, 1 (1984).
- Jaeger, J. *Climate and Energy Systems* (Wiley, London, 1984).
- Chadwick, M.J. & Lindman, N. *Environmental Implications of Expanded Coal Utilization* (Pergamon, Oxford, 1982).
- Energy Systems Program (IIASA), *The IIASA Set of Energy Models: Documentation of the Global Runs*, Prepublication Issue, IASA (1982).
- Schrattenholzer, L. *The Energy Supply Model MESSAGE*, RR-81-31, IASA (1981).
- Häfele, W. *Energy J.* **2**, 35-42 (1981).
- Nordhaus, W.D. & Yohe, G.W. in *Changing Climate*, 87-152, US National Academy of Sciences, (1983).
- Freedman, D. *J. Bus. Econ. Stat.* **1** (1), 24-36 (1983).
- Häfele, W. Lecture presented at IASA Energy Workshop, Laxenburg, Austria, June 15, 1983.
- Häfele, W. in *Nuclear Technologies in a Sustainable Energy System*, 3-20, G.S. Bauer and A. McDonald, Eds. Springer, Berlin (1983).
- Konno, H. & Srinivasan, T.N. *The Häfele-Manne Model of Reactor Strategies: Some Sensitivity Analysis*, RM-74-19, IASA (1974).
- Suzuki, A. & Schrattenholzer, L. *Sensitivity Analysis on Hydrogen Utilization Factor of the Häfele-Manne Model*, RM-74-30, IASA (1974).
- Suzuki, A. *An Extension of the Häfele-Manne Model for Assessing Strategies for a Transition from Fossil Fuel to Nuclear and Solar Alternatives*, RR-75-47, IASA (1975).
- Ho, J.K. *Holistic Preference Evaluation in Multiple Criteria Optimization*, BNL-25656, AMD-818 (Brookhaven National Laboratory, Upton, 1979).
- Sassin, W., Holzl, A., Rogner, H.H. & Schrattenholzer, L. *Fueling Europe in the Future: The Long-Term Energy Problem in the EC Countries*, RR-83-9 (IIASA, 1983).
- Rose, D.J., Miller, M.M. & Agnew, C. *Global Energy Futures and CO₂ - Induced Climate Change*, MITEL 83-015, (MIT Energy Laboratory, 1983).
- Goldenberg, J., Reddy, A.K.N., Johansson, T.B. & Williams, R.H. *Energy for a Sustainable World* (in the press).
- Landsberg, H.H. in *Carbon Dioxide Review: 1982* (ed. Clark, W.C.) (Oxford University Press (1982)).
- Thompson, M. *Policy Sciences* **17**, 321-339 (1984).
- Walters, C.J. *Adaptive Management of Renewable Resources* (Macmillan, in the press).

Where now with nuclear winter?

The US academy's predictable plea for more research should be granted if people are that worried. But the real need is for steady nerves — and atmospheric physics.

THE US National Academy of Sciences' report *The effects on the atmosphere of a major nuclear exchange* (see p.683, this issue) turns out to be what it always had to be, a first or, rather (*pace* Turco *et al.*, *Science* 222, 1283–1292; 1983), a second approximation to reality. The original account of what might happen if the atmosphere is loaded with soot from the fires ignited by 5,000 megatons of TNT in nuclear explosions is qualitatively confirmed, but hedged around with so many qualifications that a null outcome could well be compatible with the academy committee's analysis. Nuclear winter, in other words, would almost certainly be less severe, less widespread and shorter-lasting than Turco *et al.* originally declared and might even be indistinguishable from nuclear summer (which is not a pleasant prospect either). That, no doubt, is why the academy's committee has studiously refrained from using the term "nuclear winter", which is a pity; the phrase is too graphic to be ignored.

As things are, the new report will be mulled over by specialists in the field for its review of what is known of problems as different as the size-distribution of particles of smoke, the mechanisms by which the fireballs of nuclear explosions loft material to high altitudes and the ways in which climatic models may have to be refined to accommodate the needs of those who would model nuclear winter. (Cloud formation, as always, is a bugbear, what happens at the edges of a cloud a novel problem.) Latecomers in the field may, for the time being, be perplexed to know what more there can be to say, but no doubt reflection and ingenuity will enable them to think of something.

In passing, however, it must seem to many people odd that the nuclear winter subcommittee of the International Council of Scientific Unions (ICSU)'s Scientific Committee on Problems of the Environment (SCOPE), which is due to publish its own study next June, should have been able to afford time for a five-day workshop at Bellagio to draft and put out a statement which, on the face of things, will compromise the impartiality of its own work. The statement says that the prevention of nuclear war is a challenge for mankind (true), that there is "no hope" that technical innovations such as "weapons systems in space" will provide "clear superiority or significant protection" (which is probably true) and that

everybody must be "aroused" to the threat of nuclear war and to "the possibility that in some circumstances one of the results may be what has come to be called nuclear winter . . .".

The notorious ineffectualness of manifestoes apart, the danger of such statements is twofold. First, they give the impression that the issue has been decided before the study is complete. And, second, by giving needless offence to people who disagree, however wrong-headedly, with steps in their argument such as the assertion that space-based defence against ballistic missiles is impossible, they diminish their potential influence. That such a distinguished group of people (which includes Lord Zuckerman, Professor Charles Townes and Professor Abdus Salam) should be predicating its proposals for the avoidance of nuclear war on "fundamental changes in international relations, especially . . . between the Soviet Union and the United States", for all the world like old Keir Hardie socialists predicating social equality on "a change of heart", is nevertheless remarkable. The more serious problem of arms control is to avoid nuclear war even when the superpowers are daggers drawn.

This prompts one of the three general questions raised by the National Academy's deadpan text (which lets slip a flicker of emotive prose only in its last sentence, "one can ask whether even now the full range of physical consequences . . . of nuclear warfare is within our comprehension") and by the way in which the concept of nuclear winter has been used this past year. Politicians have embraced the idea (see *Nature* 13 December, p.593), usually with the best of motives. Technical people have followed suit, not for reasons that are political in the partisan sense but apparently in the belief that the threat of nuclear winter will persuade governments towards effective arms control. The risk is that such an incentive can be no more substantial than the threat. Some have further endangered their position by using the same bogey-man against extraneous targets as when Professor Paul Ehrlich told a BBC radio audience the other day that the British government's arrangements for civil defence were "nonsense", perhaps not knowing that those plans are justifiable only against a few bombs not an attack that could cause nuclear winter.

One of the technical community's admirable achievements, in what other com-

munities call the nuclear age, is its stiffness of purpose about the need somehow to contain the threat that a third nuclear weapon, then perhaps a fourth, and so on, might be used in anger. What those who work the nuclear-winter lobby overlook, or even reject outright, is that occupants of other lobbies, even the star-wars bunch, are equally steadfast in saying that their objective is to avoid nuclear hostilities. Logic-chopping in such circumstances serves little purpose (but in the 1950s it persuaded nuclear governments that the risk of genetic defects from fallout was too great to allow unrestrained nuclear testing in the atmosphere). The sad truth is that trying to bridge this gap is a plainly more formidable task than that of winning more general huzzas by identifying a hazard that others have not seen.

In the circumstances that have now developed, it is hard to know what should be done. The National Academy report asks that there should be more research. It does not say how much more. What the report has done is to identify a series of important and interesting problems in atmospheric physics that, in their own right, deserve attention. Dealing with them under the contrived rubric of nuclear winter rather than atmospheric physics will be artificial and even uneconomic. The price worth paying will depend on how deeply and generally ingrained is the belief in the certainty of nuclear winter after nuclear war. The ideal would be to spend the extra on atmospheric physics.

What should be done? Bigger and better models are not the immediate need. What the argument about nuclear winter has vividly demonstrated is that future models need better ways of dealing with physical processes in the atmosphere. After a century of research (since C.T.R. Wilson), the dynamics of cloud formation is only poorly understood, yet clouds provide the rain that cleanses the atmosphere of nuclear smoke as well as other things. Taking account of discrete clouds (as distinct from average cloudiness) in climatic models remains a puzzle. Dealing with what happens when contiguous columns in the atmosphere differ sharply in constitution (as at the edge of a cloud of nuclear smoke) is an interesting challenge. It may not be one of those "fundamental and moral challenges to all of humanity" referred to in the preamble to the SCOPE subcommittee's manifesto, but it might help people to sleep more easily in their beds. **John Maddox**

Astronomy

Draughtsmen of the constellations

from David W. Hughes

TO THE astronomically-initiated stargazer, six hundred or so of the brightest stars are dragooned into an array of constellations. For anyone who has wondered why, when and by whom this cartographic system was drafted, Archie Roy of Glasgow University provides some answers in *Vistas in Astronomy* 27, 171; 1984.

First, bear in mind that an observer living at latitude 0°N not only never sees the south celestial pole but also finds that stars with declinations between -90° and -0° never rise above the horizon. Moreover, the celestial poles are not fixed but precess about a circle of radius 23.5° every 26,000 years. It is, then, not difficult to establish the era and the domicile of the original draughtsmen. There is a patch of sky in the southern celestial hemisphere which has no ancient constellations, even though it is spattered with bright stars, and whose angular radius of avoidance is about 36° . This means that the originators of the constellations must have lived somewhere on the longitude line 36°N . And, allowing for precession, the centre of the zone corresponds to the south celestial pole of around 2,500 BC.

Why was the constellation system devised? The clues come from the work of Hipparchus (125 BC), Eudoxus (409–356 BC) and Aratus (315–250 BC). Aratus was a poet who, at the instigation of King Gonatus of Macedonia, wrote the *Phaenomena*, a poem embodying the work of Eudoxus. According to M.W. Ovenden (*Philosophical Journal* 3, 1; 1966) the daily and seasonal behaviour of the constellations discussed in this work are commensurate with a date of $2,600 \pm 800$ BC and a latitude of $36 \pm 1.5^\circ\text{N}$. But Roy's re-analysis of the poem, concentrating on its description of the positions of the celestial equator and the celestial tropics of Cancer and Capricorn, leads him to date the description to $2,000 \pm 200$ BC; Eudoxus seems to have been writing not about the sky that he could see but about the sky as it was 1,600 years earlier. Neither Eudoxus nor Aratus bothered to check that their poetic picture agreed with the Greek sky of 400–250 BC: they simply reproduced previous work. Hipparchus did check, and the discrepancies probably led him to discover polar precession.

Eudoxus was writing about people who had a sophisticated astronomical outlook which encompassed an Earth poised in space with the heavens revolving about it on a bipolar axis, although one pole was not visible. The constellation system seems to have been designed primarily as a navigational aid for sailors. Non-circumpolar constellations (those with declinations between $+0^\circ$ and -0°) always rise and set

at a specific compass point on the eastern and western horizon. A ring of constellations with the same angular distance from the celestial equator can be used to indicate a specific compass setting throughout the night and would have been a great navigational aid at a time when there was no acceptable pole star.

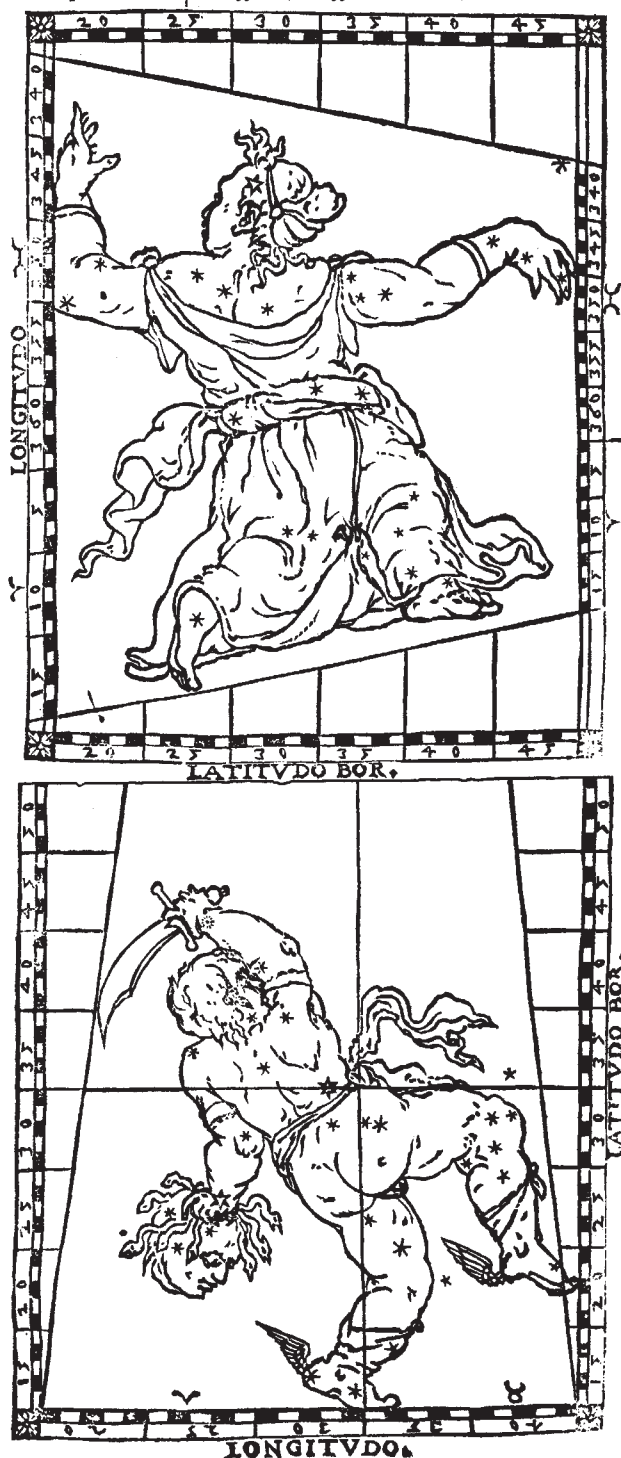
So who were the original draughtsmen? Four groups have been suspected – the Phoenicians, the Egyptians, the Babylonians and the Minoans. The Phoenicians can be ruled out because they prospered too late, from about 1500–500 BC. The Egyptians, despite their sophistication and their role as tutors to early Greek science, simply lived too far south. The Babylonians are stronger contenders: the Sumero-Akkadian people of the Euphrates region were great believers in astrology, and the movement of planets through the Zodiac was used for calendrical and religious purposes. The records left on their clay tablets leave us in no doubt that as far back as 2,100 BC they were using a system of constellations essentially similar to that given in Aratus's poem. Moreover, navigation was important to the Babylonians for their links with India and South Arabia, centred on Bahrain (ancient Dilmun). On the other hand, their sea traffic, was closer to the equator than the 'observer's latitude' derived from Eudoxus.

This leaves the Minoans, who lived on Crete and the Cyclades, and traded from there with Egypt, Greece, Cyprus,

Syria and Mesopotamia throughout the third millennia BC. Roy considers that it was the Minoans who borrowed and developed early Babylonian ideas and transformed them into a complete celestial sphere of constellations for navigational purposes.

But why was the Eudoxian sky 1,600 years out of date? A simple solution is proposed. With the complete destruction of the Minoan civilization around 1,450 BC by the volcanic explosion of Thera (Santorini), their astronomical ideas and constellations were frozen in time. □

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Andromeda (top) and Perseus by Giovanni Paolo Gallucci (1588). From George S. Snyder *Maps of the Heavens* (Andre Deutsch 1984), to be reviewed in *Nature*.

Atmospheric physics

Interaction between whistlers and radiation belt electrons

from M.J. Rycroft

ANYONE using a radio receiver during a thunderstorm is familiar with the fact that lightning produces radio waves. What is not so well known is that some of these radio waves, those at audio frequencies of a few kHz, get trapped in ducts aligned along the Earth's magnetic field and bounce back and forth ('hop') along the dipolar field lines. The dispersive nature of the medium through which the waves travel ensures that, in general, the higher frequency components travel faster than the lower. When picked up on a broad band receiver, the waves are heard as a succession of descending whistles (hence 'whistlers'), the rate of descent decreasing on successive hops. On page 740 of this issue Voss *et al.*¹ report the first satellite observations of another direct consequence of lightning flashes: the dumping of energetic electrons from the magnetosphere into the upper atmosphere¹. Such phenomena have geophysical and astrophysical relevance.

The van Allen radiation belts contain electrons and ions that are trapped by the Earth's magnetic field, accelerated to energies of some tens of keV by a variety of plasma physical processes which occur in the magnetosphere, preferentially on the night-side of the Earth, and associated with auroral substorms. Theoretical considerations of the loss of electrons from the belts have focussed on the cyclotron resonant interaction between whistlers, the frequencies of which are below both the plasma frequency and the cyclotron frequency, and energetic electrons travelling in the opposite direction along a geomagnetic flux tube. The condition for cyclotron resonance is that the electron 'feels' the wave Doppler-shifted up to its cyclotron frequency. In the equatorial plane, the phase velocity — and also the group velocity — of whistler mode waves propagating along a geomagnetic flux tube in a duct of slightly enhanced plasma density is a minimum. Thus, the equatorial plane is where cyclotron resonance occurs with electrons of the least energy.

The first observation of such a wave-particle interaction in the magnetosphere was serendipitous; during the flight of a Petrel rocket, carrying Geiger counters and launched from the Outer Hebrides, a transient increase in the intensity of energetic electrons having pitch angles between 60° and 120° was recorded (Rycroft, M.J. *Planet. Space Sci.* 21,239; 1973). The increase, by a factor of 20 above the quasi-steady intensity observed throughout the remainder of the flight, occurred in 0.8 seconds and was simultaneous for both >45 keV and >110 keV electrons. About

half a second later a two-hop whistler was recorded on the ground. During the period of enhanced electron intensity, the entire duration of which was only about six seconds, four-, six- and eight-hop whistlers were also received. From an analysis of their spectrogram, it was concluded that the whistlers were ducted through the magnetosphere along the $L = 3.3 \pm 0.1$ field line. The electron density in the equatorial plane, N , was found to be $330 \pm 10 \text{ cm}^{-3}$, a value that is characteristic of conditions within the plasmopause.

It was suggested that the association of whistler and electron intensity was not coincidental but the result of a cyclotron resonant interaction between energetic electrons and whistler mode waves moving in opposite directions along the $L = 3.3$ flux tube. For gyroresonance on this flux tube at the equator, the parallel component of energy of the electrons is 25 keV at 3 kHz in the whistler band, or 100 keV at 1 kHz below it. A possible explanation is that energetic electrons of a sufficiently anisotropic pitch-angle distribution-function and associated with electrons injected during an earlier auroral substorm become unstable, through cyclotron resonance instability, when they drift into the high density plasmasphere. The instability causing the burst of electron precipitation was believed to be triggered by the half-hop whistler propagating across the equator. The whistler was amplified during the interaction; it was later observed, above the

background noise level, as a two-hop whistler.

Voss *et al.* have measured this wave-particle interaction by sophisticated solid-state instrumentation cooled to 150 K aboard the S 81-1 satellite in a polar orbit at 230 km altitude. They show several examples of one-hop whistlers, observed on the ground in Antarctica, that are associated with bursts of precipitating electrons. The flux of precipitating electrons is observed to rise by a factor of up to 100 within 0.2 seconds. Because the cyclotron resonance interaction reduces the pitch angle of energetic electrons trapped in the magnetosphere, their mirror points descend below the satellite altitude. For cyclotron resonance in the equatorial plane of the $L = 2.2 \pm 0.1$ flux tube, N is estimated to be 3200 cm^{-3} ; waves with a frequency of 4 kHz resonate with 150 keV electrons. Such energetic particles can penetrate the atmosphere down to a height of 80 km, where they cause a significant amount of secondary ionization. This can affect the characteristics of very low frequency radio waves propagating in the Earth-ionosphere waveguide.

The overall efficiency of whistlers in causing the loss of the van Allen radiation belt electrons is still not known. However, with a globally-averaged rate of about 100 lightning discharges per second (Brookes, C.E.P. *Geophys. Mem.*, London 24; 1925) there is considerable scope for further experimental studies of this wave-particle interaction. Such studies are also of relevance to other magnetospheres in the Universe, and to the loss of charged particles from plasma devices in any Earth-bound laboratory that relies on magnetic mirrors for plasma containment. □

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A CORE sample, obtained in the course of test-drilling from a North Sea oil rig belonging to Marathon Oil, produced this 150-million year old fossil of the front half of the fish *Tharsis dubius*, now in the British Museum of Natural History.



Tropical ecology

Long-term rainforest studies

from Jared M. Diamond

MOST biologists have their permanent homes in the temperate zones. The most species-rich biological communities are in the tropics, the rainforests of which are often described by stereotypes such as 'seasonally constant' and 'complex'. Dr. Harry Bell of the University of New England in Armidale, Australia is responsible for one of the few uninterrupted and intensive ecological studies by biologists resident in the tropics and his recent papers provide a wealth of quantitative information for tropical bird communities of New Guinea.

Following a series of studies that began in 1964, Bell devoted 1975–1978 to a census of the 165 bird species on a 2.5 hectare plot of rainforest^{1,2} and on a nearby 1-km transect across savanna³. He estimated the diet, body weight, population density, vertical distribution, and the seasonal changes in status of each species.

Although New Guinea is near the equator and 80 per cent of the rainforest bird species show no significant seasonal changes in abundance, their breeding is as markedly seasonal as in the temperate zones, although geared to changes in rainfall rather than day-length^{1,3,4}. Most carnivores and omnivores breed in the drier season, before the onset of the heavy rains that would damage their nests¹. Most ground-feeders breed in the wetter season, when the ground is soft and food more easily extracted^{1,4}. The grass warbler *Cisticola exilis* starts calling and displaying within three days of a single rain, and ceases again in a few days if there is no more rain³.

Compared with temperature-zone birds, those of New Guinea have low nesting success, low recruitment of young, and are long-lived^{1,4,5}. Open nests contain only 1.4 eggs on average (4–6 in temperate zones), and an astonishingly high number (88 per cent) of the clutches fail¹. Of young paradise kingfishers that leave the nest, most disperse, starve and die in their first year without having obtained a territory⁴. Conversely, annual mortality of adults is only 10–20 per cent⁵.

Bell estimates there to be 69 birds per hectare, between 3–30 times more than in most temperature-zone forests¹. Bird biomass is about 5 kg per hectare. Even compared with other tropical areas, New Guinea has a high species diversity and biomass of obligate frugivores (especially, large pigeons and parrots) — probably an evolutionary result of the paucity of fruit-eating mammals on the island.

Some of Bell's observations highlight two under-appreciated factors in habitat selection by rainforest species. First, some insectivores of the dark understory with

large eyes refuse to cross even small sunlit paths¹, whereas other species of the forest canopy seem to need strong sunlight. Second, the parrot *Pseudeos fuscata* will never cross a mountain pass through which a stream runs, in its daily commuting of up to 50 km from roosting to feeding sites across the lofty Central Cordillera of New Guinea¹.

Bell's papers abound with examples of the complex interspecies relations for which the tropics are famous. Kingfishers and pygmy parrots excavate their nests in arboreal termite nests^{4,6,7}. Different kingfisher species use different-sized nests, and the density of termite nests probably limits kingfisher abundance in second growth⁷. Ground pigeons that swallow pebbles, and thereby can grind up hard seeds in their stomachs, collect seeds defecated or regurgitated intact by frugivorous bird species with weak-walled stomachs⁶. The male and female of the flycatcher *Arses telescopthalmus* are each more similar ecologically to other fly-catcher species than to one another. Compared with the female, the male has longer and more curved claws and a more slender bill, for gleaning insects off understory vertical trunks and vines, whereas the female has a longer tail, wider

bill, and more rectal bristles, for catching insects in midair⁸.

One of the most interesting examples of group behaviour involves the babbler *Pomatostomus isidori*¹. This species lives in groups of about eight, which jointly build one nest, roost in the nest, and rear and feed one or two young. They forage together and exchange incessant contact calls and an all-clear 'yahoo' call, with one individual acting as leader and giving a distinctive 'leader call'. The babbler flock is followed by rusty pitohuis (*Pitohui ferrugineus*), and both the babblers and pitohuis are followed by bird species of several other families (including birds of paradise), all of which mimic the babbler's and pitohui's rusty colour, and some of which also mimic the calls.

Until recent decades, tropical ecology was regarded as hopelessly complex, and graduating ecologists were advised to study simpler communities like the desert and the arctic. Bell's papers set new standards for quantitative long-term tropical work and show that rainforest studies are complex and fascinating but not hopeless. □

1. Bell, H.L. *Emu* 82, 7; 24; 65; 143; 217; 256 (1982).
2. Bell, H.L. *Emu* 84, 142 (1983).
3. Bell, H.L. *Ibis* 124, 252 (1982).
4. Bell, H.L. *Corella* 4, 113 (1980).
5. Bell, H.L. *Corella* 6, 77 (1982).
6. Bell, H.L. *Aust. Bird Watcher* 10 209 (1984).
7. Bell, H.L. *Ibis* 123, 51 (1981).
8. Bell, H.L. *Aust. J. Ecol.* 7, 137 (1982).

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Biotechnology

Cancer toxin genes cloned

from Karol Sikora

CLINICAL research into cancer treatment badly needs new leads. The major stumbling block is the poor discrimination of systemic agents for common solid tumours. Some immunological mediators are known to have specific anti-tumour effects but the mechanisms involved are poorly understood. Ultimately, it is only through molecular cloning that the individual proteins will be characterized and their functions elucidated. Interferons, the first to be cloned, are now undergoing extensive, if rather disappointing, clinical trials¹. On page 721 and 724 of this issue the cloning of two other molecules of great clinical potential, lymphotoxin and tumour necrosis factor, is reported^{2,3}.

The discovery of cancer toxins dates back at least to 1891 when a New York physician, William Coley, began giving patients mixtures of toxins from various bacteria⁴. His rationale was that cancers sometimes regressed in patients after a severe bacterial infection. The dramatic tumour responses he saw were later reported with evangelical zeal⁵ and the whole sub-

ject of the anti-tumour effects of bacterial endotoxins heralded in the era of active immunotherapy. But the bacterial products themselves were not found to destroy tumour cells *in vitro*. This paradox was resolved by the discovery that endotoxins, when injected into suitably primed animals, stimulate macrophages to produce a 'tumour necrosis factor' (TNF) that causes haemorrhagic necrosis in tumors⁶. But even before this, tumour immunologists had demonstrated that sensitized lymphocytes could also destroy tumour cells through soluble mediators — the lymphokines, including lymphotoxin (LT)⁷.

Antibodies to partially purified TNF and LT provided evidence of structural differences between the two cancer toxins, both of which are able to distinguish between tumour cells and normal cells, and destroy only the former^{8,9}. But although an intriguing new pair of selective tumour-destroying agents had been discovered, experimentalists were plagued by their impurity. Batch-to-batch variation, tedious bioassays and the lack of molecular characterization precluded further analysis. Indeed,

the observations were dismissed by some as an over-optimistic interpretation of confused data.

Against the background of doubt, the use of sophisticated biotechnology has now unravelled the structure of both TNF and LT. The gene for TNF was cloned from the human promyelocytic leukaemia line HL60, which produces large quantities of TNF upon stimulation. Amino acid sequence data from tryptic peptide analysis led to the construction of a 42-base oligodeoxynucleotide that was used to extract TNF cDNA from a cDNA library. From the sequence of the cDNA it is clear that TNF is made as a 157-amino acid precursor from which the amino-terminal 76-amino acid segment is cleaved prior to secretion of TNF. The cleaved fragment may well represent an unusually long signal peptide and contains arginine and lysine dipptide pairs of the kind often associated with the release of physiologically-important peptides from precursor molecules¹⁰. The TNF produced when the cDNA was expressed in *Escherichia coli* was tested for biological activity; tumour necrosis is demonstrable with microgramme quantities.

LT was purified from a phorbol ester-stimulated human lymphoblastoid cell line, its amino-terminal 155 amino acids were determined by microsequencing and a corresponding oligodeoxynucleotide was synthesized. This long probe was used to isolate a cDNA clone from which the complete LT sequence was derived. Again, a signal sequence is found, in this case 34 amino acids long. LT produced from the cDNA in *Escherichia coli* has clear anti-proliferative effects on several human tumour cell lines but not on normal cells. Furthermore, it destroyed a murine sarcoma *in vivo*.

Both molecules can kill tumour cells. Can we learn anything of their structure-activity relationships? It turns out that there is considerable similarity between the sequences of TNF and LT. Of the 157 amino acids of TNF, 44 are matched in LT when suitable gaps are introduced. Many more amino acids show conservative changes. There are two well conserved regions within the molecules in addition to the hydrophobic carboxy-termini. One conserved region perhaps binds to a cellular receptor, the others may effect cell destruction. Many intriguing questions can now be addressed. What is the basis for

selective tumour cell destruction? Do these toxins have to be released in the vicinity of a tumour or are they effective systemically? Is there species-specificity? Now that the genes are cloned, material will be available for these studies.

What of the clinical potential of the toxins? Several groups have cloned, or are on their way to cloning, the DNA for one or both toxins and clinical trials will start soon. Animal experiments are in progress to determine the pharmacokinetics of tumour destruction. Most of the early animal work used crude extracts injected into the tumours but the clinician will be more interested in systemic therapy

because of the widespread nature of metastatic disease. As with the interferons, there may be profound side effects¹¹. In fact, the symptoms of many diseases are probably mediated through the release by the immune system of proteins such as TNF and LT. The elucidation of the precise structure of these two molecules and their production in a pure form will stimulate renewed interest in their clinical exploitation and in the biological mechanisms that control cancer. □

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Molecular biology

Upstream and downstream control of eukaryotic genes

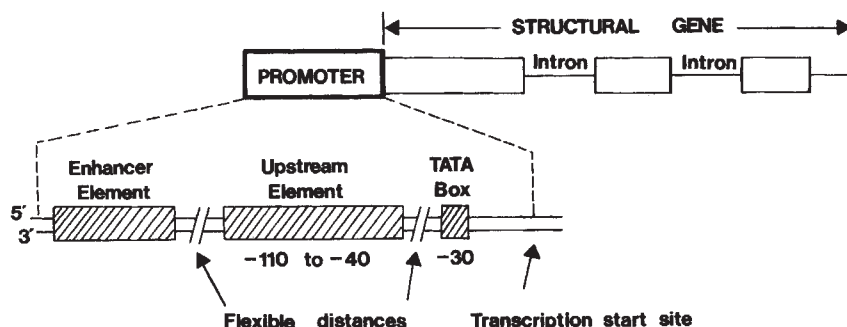
from Tim Reudelhuber

How does a specialized cell selectively express only a small subset of the tens of thousands of genes at its disposal? This question is of fundamental importance in molecular biology and may also have practical importance in such fields as medicine and agriculture. In prokaryotes, transcription is controlled by regions of DNA on the upstream, or 5', side of structural genes. These regulatory or promoter regions are composed of a set of DNA sequence elements whose function, in most cases, has been clearly demonstrated. As a logical extension of this knowledge, the attempt to identify transcriptional control elements in eukaryotes has also been focused, with considerable success, on the DNA sequences upstream of structural genes. But two recent reports in *Cell* suggest that the human adult β -globin gene may also contain a crucial control element downstream of its transcription start site^{1,2}.

The upstream control elements of eukaryotic genes are classified into three types based on their function, their sequence characteristics and their position relative to the start site of transcription (see Fig. and ref. 3). One type, termed a TATA box element (because the sequence is fre-

quently TATA), is involved in fixing the start site of transcription to a point 30 base pairs downstream from its own position. A second type, the upstream element, is a broad class of sequences, at a variable distance from the transcription start site, that seems to be important in determining the level of transcription. These elements have variously been referred to as G-C rich elements and even CCAAT boxes (again because of their sequence). Finally, enhancer elements, which were first reported in mammalian viruses, seem to be able to stimulate gene transcription from considerable distances and have been postulated to be tissue-specific modulators of transcription for some mammalian genes (see for example refs. 4, 5).

The evidence that a human globin gene is, in part, controlled by a downstream element arose from attempts to define the DNA sequences responsible for the differential expression of human α -globin and β -globin genes. The adult α -globin gene is expressed throughout fetal and post-natal life whereas the members of the β group are expressed during development in the order ϵ (embryonic), γ (fetal) and adult β globin. The controlling sequences for this differential expression were investigated by



Features of eukaryotic protein-coding genes. Protein-coding sequences (open boxes) and interspersed introns comprise the structural gene, upstream of which is a promoter region containing three types of control elements. (Courtesy A. Wildeman.)

1. Sikora, K. (ed) *Interferon and Cancer* (Plenum Press, New York 1983).
2. Gray P.W. *et al.* *Nature* **312**, 721 (1984).
3. Pennica, D. *et al.* *Nature* **312**, 724 (1984).
4. Coley, W.B. *Trans. amer. Surg. Assoc.* **12**, 183 (1984).
5. Nauts, H.C., Fowler, G.A. & Bogatko, F.H. *Acta. Med. Scand.* **276 Supp.** 5 (1953).
6. Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. & Williamson B. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3666 (1975).
7. Granger, G.A. & Kolb, W.P. *J. Immunol.* **101**, 111 (1968).
8. Helson, L., Green, S., Carswell, E. & Old, L.J. *Nature* **258**, 731 (1975).
9. Ruddle, N.H. & Waksman, B.H. *J. exp. Med.* **128**, 1267 (1968).
10. Gubler, U., Seeburg, P., Hoffman, B.O., Gage, L.P. & Udenfriend, S. *Nature* **295**, 206 (1982).
11. Smedley, H.M., Katrak, M. & Sikora, K. *Brit. med. J.* **286**, 262 (1983).

Charnay *et al.* by means of introducing the cloned genes into a mouse erythroleukemia (MEL) cell line¹. If terminal differentiation of MEL cells is induced by various agents *in vitro*, there is a concomitant activation of transcription from the endogenous α -globin and β -globin genes and from the introduced cloned human β -globin gene, but not from the introduced cloned human α -globin gene (which seems, inexplicably, to be expressed constitutively before and after differentiation).

The striking finding reported by Charnay *et al.*¹ is that a hybrid of the human α -globin upstream promoter region and the β -globin structural gene sequences is inducible in the same manner as the complete β -globin gene. An identical finding is reported by Wright *et al.* from experiments with hybrid genes that consist of fusions between the promoter of either the non-inducible human fetal (γ) globin gene or a mouse histocompatibility antigen (H-2K^{bml}) gene and human adult β -globin structural gene sequences. Again, upon introduction into MEL cells, the hybrid genes were β -globin-like in their induction. These results suggest that the β -globin gene, which has a full complement of functionally important upstream promoter elements⁶, also harbours an element within the structural gene that is crucial for appropriate expression in erythroid cells.

With these findings, the human β -globin gene joins a small but growing number of eukaryotic genes that have been shown to have regulatory sequences downstream from the start site of transcription. The *Xenopus* 5S ribosomal RNA gene (which is transcribed by a different RNA polymerase) also has a regulatory element within the domain of its structural gene⁷; some mouse immunoglobulin genes contain an enhancer element within an intron⁴; and the chicken thymidine kinase gene has an intragenic control element (although it is not yet known whether this control is at the transcriptional level)⁸.

Could gene regulation be one of the functions of the gene-splitting introns of eukaryotes? Circumstantial support for this hypothesis comes from the finding that the introns of many coordinately-controlled and neurone-specific genes in the rat brain contain some sequences in common⁹. In any case, it is apparent that despite the tremendous recent progress in determining the control elements of eukaryotic genes, we have still a lot to learn. □

1. Charnay, P. *et al.* *Cell* **38**, 251 (1984).
2. Wright, S., Rosenthal, A., Flavell, R. & Grosfeld, F. *Cell* **38**, 265 (1984).
3. Chambon, P. *et al.* *Recent Progress in Hormone Res.* **40**, 1 (1984).
4. Picard, D. & Schaffner, W. *Nature* **307**, 80 (1984).
5. Walker, M.D., Edmund, T., Boulet, A.M. & Rutter, W.J. *Nature* **306**, 557 (1983).
6. Dierks, P. *et al.* *Cell* **32**, 695 (1983).
7. Schlissel, M. & Brown, D. *Cell* **37**, 903 (1984).
8. Merrill, G.F. *et al.* *Mol. and cell. Biol.* **4**, 1777 (1984).
9. Milner, R.J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 713 (1984).

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Endocrinology

Enlightenment and confusion over steroid hormone receptors

from R.J.B. King

STEROID hormones combine with specific receptor proteins to form a complex that binds to cell nuclei, thereby switching on messenger RNA (mRNA) synthesis from specific genes. That statement could have been made nearly twenty years ago; the intervening years have been spent in sorting out the nuts and bolts of the model. Major questions have been: what is the relationship of the cytoplasmic '8S' receptor protein (if it exists) to the smaller nuclear receptor; what is the nuclear acceptor for the hormone-receptor complex; how does the complex switch on the genes; and how do different hormones influence the same cellular function?

Many of the difficulties encountered in attempts to answer these questions can be ascribed to two practical problems. First, receptors have, until recently, only been detectable by the use of radioactive steroids, so that in most cases only the functionality of the steroid-binding domain of the receptor has been followed. Second, there has often been no certain way of testing the biological relevance of the molecular interactions being studied.

In the case of the glucocorticoid hormones, the latter problem has been partly resolved by the isolation of variant mouse lymphoma cell lines that are abnormally unresponsive to glucocorticoids¹. The variant cells fall into three categories: those that are deficient in the cytoplasmic receptor (r^-); those that are defective in nuclear transfer (nt^-); and those with increased nuclear transfer (nt^+). These variants have enabled some correlations between receptor properties and biological function to be made. Work on the glucocorticoid receptor has also been helped by the molecular dissection of mouse mammary tumour virus (MTV), which is transcribed into RNA when induced by glucocorticoids. A combination of studies involving the construction of chimaeric genes, a range of viral deletion mutants and the binding of purified receptor to specific DNA constructs has suggested that glucocorticoid receptor switches on MTV transcription by binding to specific sequences within the 'long terminal repeat' of MTV²⁻⁴. In other genes, too, the evidence is pointing to a region of DNA upstream of the mRNA initiation site as the acceptor site for steroid receptors. Interestingly, there are additional binding sites for the receptor. They are within the structural gene and of unknown function.

If all steroid receptors bind to some upstream gene region, it could explain how one biological function can be produced by different steroids through the appropriate

receptors. Glucocorticoid and progesterone receptors have recently been shown to bind *in vitro* to overlapping regions of the MTV and chicken lysozyme DNA^{6,7}, and there is increasing comment about consensus sequences able to recognize different receptors. Moreover, unpublished data from my own laboratory suggest that the long terminal repeat of MTV, which classically responds only to glucocorticoids, responds to androgens after it is experimentally transferred to cells that carry androgen receptors.

As to the shortcomings of relying on radioactive ligands, it has long been accepted wisdom that they would largely be overcome by the production of good antibodies to receptors. This has proved to be correct but only at the cost of raising both tempers and more questions. Where monoclonal antibodies have been generated unequivocally against the steroid-binding subunit of a receptor, lack of cross-reactivity with other classes of steroid receptor has been established and, in some cases, receptor genes and their mRNAs identified. Thus, on page 779 of this issue, the groups of Yamamoto and Gustafsson describe the use of MTV, the lymphoma variants and monoclonal antibodies to a glucocorticoid receptor, not only to identify the receptor gene and its mRNA but also to characterize further the lymphoma variants⁸. Perhaps their most interesting result is that even the r^- cells produce a normal-sized mRNA from the receptor gene, albeit in reduced quantity. Why should there be any mRNA in the r^- cells? The authors suggest that the r^- (and nt^+) cells produce a non-functional 'receptor' protein.

A 'receptor' that does not bind hormone — in this case progesterone — has also been identified in chick oviduct cells and its cDNA has recently been isolated in O'Malley's laboratory. A monoclonal antibody against the B-subunit of the receptor was used to identify the gene product. The antigen is similar to the progesterone-binding domain both with respect to size and peptide map. On the other hand, Baulieu's group has a monoclonal antibody to chick oviduct progesterone receptor which recognizes a protein that does not bind steroid, shows no similarity to the steroid-binding domains and which is claimed to be common to all classes of receptor¹⁰. O'Malley's group, however, has since suggested that this protein is an artefact¹¹.

Thus, there are at least three candidate components of the '8S' receptor — a steroid-binding subunit and two non-

binding subunits one of which is similar, and the other dissimilar to the binding subunit. Additional examples exist of monoclonal antibodies raised against a partially purified glucocorticoid receptor¹² or an oestradiol receptor (in my own laboratory) that do not react with steroid binding subunits. Confusion abounds. But if the nuclear rather than cytoplasmic location of unoccupied oestradiol receptor is verified¹³, the '8S' receptor may anyway be an artefact.

My own feeling is that, given the impure nature of the receptor preparations used to raise the monoclonal antibodies and the general ability of steroid-binding subunits to react non-specifically with a wide range of macromolecules, the characterization of the antibodies should be closely scrutinized. If the antibody reacts directly and unequivocally with a steroid-binding protein, then valid deductions are in order; if this criterion is not satisfied, data from the system should be interpreted with caution. My current view is that the texts on receptor structure will require rewriting but it would be

foolhardy to start just yet. On the other hand, work on gene isolation of the type described in this issue of *Nature* represents a major advance towards our understanding of steroid hormone action. □

1. Sibley, C.H. & Yamamoto, K.R. in *Monographs on Endocrinology, Vol. 12. Glucocorticoid Hormone Action* (eds Baxter, J.D. & Rousseau, G.G.) 357 (Springer, Berlin, 1979).
2. Payvar, F. *et al. Cell* **35**, 381 (1983).
3. Pfahl, M., McGinnis, D., Hendricks, M., Groner, B. & Hynes, N.E. *Science* **222**, 1341 (1983).
4. Ringold, G. *et al. in Progress in Cancer Research and Therapy, Vol. 31. Hormones and Cancer 2* (eds Bresciani, F., King, R.J.B., Lippman, M.E., Namer, M. & Raynauld, J.-P.) 7 (Raven, New York, 1984).
5. Parker, M. *Nature News and Views* **304**, 687 (1983).
6. Renkawitz, R., Schütz, G., von der Ahe, D. & Beato, M. *Cell* **37**, 503 (1984).
7. Von der Ahe, D., Janich, S., Scheidereit, C. & Beato, M. *Nature* (in the press).
8. Miesfeld, R. *et al. Nature* **312**, 779 (1984).
9. Zarucki-Schulz, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 6358 (1984).
10. Joab, I. *et al. Nature* **308**, 850 (1984).
11. Birnbaumer, M., Bell, R.C., Schrader, W.T. & O'Malley, B.W. *J. biol. Chem.* **259**, 1091 (1984).
12. Westphal, H.M., Mugele, K., Beato, M. & Gehring, U. *EMBO J.* **3**, 1493 (1984).
13. Schrader, W. *Nature News and Views* **308**, 17 (1984).

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Earth sciences

Suspect Sm-Nd whole-rock ages

from M.J. Bickle

WELL-preserved volcanic and sedimentary rocks in Archaean greenstone belts provide a unique record of surface and magmatic processes during the first half of the history of the Earth. Unravelling stratigraphic relationships in the structurally complex greenstone terrains is the first prerequisite to their interpretation. This depends largely on isotopic methods of geochronology, particularly the decay of uranium to lead and of samarium to neodymium in whole-rock samples. Although most Sm-Nd ages determined in whole-rock samples agree well with ages derived by other methods, some significant discrepancies have recently emerged, highlighted this year by reports of markedly different ages for the volcanic succession of Kambalda in Western Australia^{1,2}. The age of the Kambalda sequence is important not only because of its tectonic significance but also as a guide to the search for nickel ores.

For Kambalda, Claoué-Long *et al.*¹ obtain a whole-rock Sm-Nd isochron of $3,262 \pm 44$ Ma (2 σ errors) on samples of komatiite, high-Mg basalt and tholeiitic basalt. This is about 400 Ma older than, both a Pb-Pb age of $2,720 \pm 105$ Ma on the same volcanic rocks and model Pb ages of $2,840 \pm 35$ Ma on the associated iron-nickel-sulphide ores obtained by Roddick². Until recently, the Sm-Nd age would have been accepted without question because it satisfies the normal criteria. In particular, the samples include only basalts and magnesium-rich komatiite lavas, and not the felsic volcanics and

intrusives that may not be cogenetic but were included in a previous study³.

U-Pb systematics in volcanic rocks are relatively easily altered and, as discussed by Roddick², the Pb-Pb whole-rock age on the volcanics may be reset by metamorphism. The model Pb ages of $2,840 \pm 35$ Ma on the iron-nickel-sulphide ores are less easy to reconcile with the Sm-Nd age. Field, petrological and geochemical criteria⁴ are consistent with separation of the iron-nickel-sulphide ores as an immiscible sulphide liquid during emplacement of the high-temperature komatiite lavas; alteration of the Pb isotopic compositions of $3,260$ Ma Pb-rich, U-poor sulphide ore to lie near the $2,800$ Ma point on the geochron is not easy to envisage.

A major difficulty is that the age discrepancy is not readily testable by alternative methods as there are few independent constraints on the age of the Kambalda sequence. The only good date available from the Kambalda area is a minimum age set by an intrusive $2,820 \pm 15$ Ma granodiorite⁵. Stratigraphic correlation of the sequence at Kambalda with surrounding areas is uncertain in this structurally complex and poorly exposed terrain; indeed, this is an area where geologists wish to use isotope geochronology to test the stratigraphy.

The only piece of evidence seriously to challenge the Sm-Nd result is the Pb isotopic composition of the iron-nickel-sulphide ore, but model Pb age calculations are essentially empirical and do not

provide internal tests of consistency. Even so, may the Sm-Nd be in error? A few recent studies have revealed potential problems with Sm-Nd geochronology that may be partially explained by the emplacement of high-temperature komatiite lavas. A sequence of basalts and komatiites in the Abitibi Belt, Canada, gives a whole-rock Sm-Nd isochron of $2,826 \pm 64$ Ma, but the age of the rocks is well constrained to less than $2,697 \pm 1.1$ Ma by a U-Pb zircon age on stratigraphically-underlying felsic volcanics⁶, suggesting that this systematic error in the Sm-Nd age results from heterogeneity of initial Nd isotopic ratios of the source⁶. Dupré *et al.*⁷ record variable Sm/Nd ratios within an individual flow elsewhere in the Abitibi Belt and conclude that the variation results from contamination due to melting and assimilation of the underlying flow. Thus, both source heterogeneity and contamination could lead to systematic errors in Sm-Nd whole-rock ages.

Where the initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratio and Sm/Nd ratio correlate, a linear relationship between the two parameters will be preserved on the isochron diagram but age calculation will be systematically in error. Correlation of any initial daughter isotope ratio with parent/daughter ratio may occur in any whole-rock geochronological method, but the very similar geochemical behaviour of the rare-earth Sm and Nd limits the dispersion of Sm/Nd ratios and makes the age calculations sensitive to small systematic differences in initial Nd isotopic composition. Both mantle source heterogeneity and contamination can lead to initial $^{143}\text{Nd}/^{144}\text{Nd}$ and Sm/Nd ratios that are relatively well correlated.

If the $3,260$ Ma age of the Kambalda suite is a result of source heterogeneity, the mantle source regions for the lavas must have had a long history of variable depletion by $2,800$ Ma. Whether petrogenetic processes of melting and fractionation could erupt lavas with unchanged, or systematically changed, Sm/Nd ratios is open to question.

The likelihood of contamination of the unusually high temperature komatiite lavas has been given new emphasis by recent theoretical and experimental modelling of their emplacement. Huppert *et al.*⁸ have suggested that the flows may melt up to 10 per cent of their volume of underlying rock. This process may be the cause of the deep channels at Kambalda, which are cut into the underlying basalt and

1. Claoué-Long, J.C., Thirlwall, F.M. & Nesbitt, R.W. *Nature* **307**, 697 (1984).
2. Roddick, J.C. *Geochim. cosmochim. Acta* **48**, 1305 (1984).
3. McCulloch, M.T. & Compston, W. *Nature* **294**, 322 (1981).
4. Gresham, J.J. & Loftus-Hills, G.D. *Econ. Geol.* **76**, 1373 (1981).
5. Compston, W. *History of the Crust and Mantle at Kambalda using Isotopic Age Determinations* (Western Australian Institute of Technology, Perth, 1980).
6. Cattell, A., Krogh, T.E. & Arndt, N.T. *Earth Planet. Sci. Lett.* (in the press).
7. Dupré, B., Chauvel, C. & Arndt, N.T. *Geochim. cosmochim. Acta* (in the press).
8. Huppert, H.E., Sparks, R.S.J., Turner, J.S. & Arndt, N.T. *Nature* **309**, 19 (1984).
9. Leshar, C.M. thesis Univ. Western Australia (1983).

filled with iron-nickel-sulphide and komatiite⁹. Similar contamination by melting wall rock during ascent of magmas through the crust is equally likely.

Until criteria to choose between mantle heterogeneity contamination or complete resetting of the Pb isotopes are found, or an independent age constraint is available on Kambalda, the uncertainty in the interpretation of the Sm-Nd age will remain. Resolution of the problem may have

important implications either for the degree of heterogeneity and depletion of the Archaean mantle of the extent of contamination of komatiitic lavas. The latter may necessitate reinterpretation of the constraints komatiite lavas place on the chemistry of the Archaean mantle. □

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Ecology

Why be an evergreen?

from Peter D. Moore

CONSIDERING their importance to plants, it is surprising that we do not know more about leaves. What, for example, determines leaf size and what is the adaptive significance of size and longevity in leaves? Presumably the evolution of the evergreen habit conveys certain advantages upon a plant under given environmental conditions. What they may be is the question raised by three new studies¹⁻³.

It has long been recognized that there is a relationship between leaf form and climate⁴ but the causes underlying the relationship have proved elusive. Parkhurst and Loucks⁵ constructed a model of optimal leaf size on the assumption that the optimum under given conditions is that which provides the maximum efficiency of water use, that is the maximum uptake of CO₂ per unit of water lost. Thus, large leaves should be favoured only under warm conditions with low light intensity. They tested their model, which accounted for seven independent variables, and found it to match well with observed data. It has subsequently been shown to operate in a variety of situations including the Galapagos Islands, where there is a positive relationship between the average leaf size of a plant community and rainfall, and where leaves in the herbaceous layer of a structured community are larger when the canopy layer is denser⁶.

Although leaf size has been shown to follow certain rules, at least on a large scale consideration, leaf longevity has proved less amenable to explanation. Hamann found it difficult to explain the distribution pattern of evergreen species on the Galapagos Islands; no advantages over their deciduous counterparts were immediately apparent, though evergreens appear to be more frequent in sites with more prolonged drought⁶.

Explanations of the advantages of the evergreen habit have tended to fall into two camps. The main advantage is claimed to be energetic, the evergreen leaf being capable of taking advantage of short periods of suitable conditions within a generally unfavourable period of, for example, cold or drought. The alternative explanation proposes that long-lived

evergreen leaves act as storage systems for nutrients in short supply, such as nitrogen, phosphorus and potassium⁷, and that it is therefore of selective advantage to be an evergreen in low nutrient environments such as bogs and heathland.

In their recent review of the energetic advantages of evergreens in winter conditions, Larcher and Bauer⁸ comment on the scarcity of data. In general they feel that winter photosynthesis may be significant in Mediterranean and oceanic areas but it unlikely to be of importance in regions with severe winters. Robertson and Woolhouse¹ have now shown that a herbaceous species, the cotton sedge *Eriophorum vaginatum*, a major dominant species on the upland areas of northern and central Europe, retains some leaves through the winter which are capable of activity very early in the growing season. These leaves can be operating at up to half-maximal rates before the new season's crop of leaves has been produced, and they continue to photosynthesize throughout the growing season. Interestingly, however, the leaves of *E. vaginatum* also act as reservoirs for elements such as phosphorus and potassium⁹, so a combination of advantages could be involved here.

These observations apply to oceanic

environments but some comparable data have emerged from a distinctly continental environment in Utah in the United States. Nowak and Caldwell² monitored the photosynthetic rates of two introduced grasses, *Agropyron desertorum* and *A. spicatum*, during winter when the soil is permanently frozen and snow cover is intermittent. Many leaves of these grasses survive the winter and show a positive carbon gain during those periods when they are not covered with snow. Such observations provide fuel for the energetic advantages of evergreen leaves.

At the other end of the climate spectrum are the forests of north-east India. Shukla and Ramakrishnan find that the majority of early successional trees are of evergreen habit, but that the leaves are generally short-lived (usually less than 200 days) whereas late successional species, have a deciduous habit and their leaves may last 300 days. In that case, evergreen habit could be of advantage in providing fast growth, especially when combined with a rapid leaf turnover, enabling new leaves to be produced in the most appropriate positions to take advantage of the light.

The impression gained from these varied studies in a range of climatic regimes is that there is no simple, general reason why the evergreen habit can be of selective advantage. Both nutrient conservation and energetic opportunism may give a plant the necessary competitive edge when facing an unfavourable period. It clearly pays some plants to keep their leaves. □

1. Robertson, K.P. & Woolhouse, H.W. *J. Ecol.* **72**, 685 (1984).
2. Nowak, R.S. & Caldwell, M.M. *Photosynthetica* **18**, 192, (1984).
3. Shukla, R.P. & Ramakrishnan, P.S. *New. Phytol.* **97**, 697 (1984).
4. Raunkiaer, C. *The Life Form of Plants and Statistical Geography* (Clarendon Press, 1934).
5. Parkhurst, D.F. & Loucks, O.L. *J. Ecol.* **60**, 505 (1982).
6. Hamann, O. *Biotropica* **11**, 101 (1979).
7. Rundel, P.W. & Parsons, D.J. *Am. J. Bot.* **67**, 51 (1980).
8. Larcher, W. & Bauer, H. *Encyclopedia of Plant Physiology* **12A**, 403 (1981).
9. Goodman, H.J. & Perkins, D.F. *Nature* **184**, 467 (1959).

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100 year ago

PRONUNCIATION OF CHINESE NAMES

SOMEWHAT after date, I beg to return to the subject of Anglo- and Franco-Chinese orthography, referred to in *NATURE* vol. xxx. p. 592. In a short paper of mine published in the *Proceedings of the Royal Geographical Society*, vol. xxii, No. 6, 1877, I alluded to the desirability of a uniform or fixed "Roman equivalent" for Chinese characters standing for names of place, &c. To my mind the Italian vowels, &c. come nearest to the sounds of the Chinese characters. *Tung-King*, meaning "Eastern Capital," is the usually accepted form of *Tonquin*, or *Ton-Kin*, the terminal *g* being

but slightly sounded. *Shang-hai*, the "Upper Sea," or the place "of going up to the sea," should be pronounced with the *g*, and is so spoken (Shanghai) by English and American authorities. Dr. Wells Williams has, I believe, in manuscript a standard Chinese Gazetteer of the world, in which all proper names likely to be used in telegraphy, newspapers, &c., are smoothly transliterated into Chinese characters. For translation from Chinese it is very necessary to adopt some such plan as Dr. Hunter has suggested for Indian names. Although his plan has come too late into the field to induce people to spell Calcutta as Kolkata, this is hardly the case as yet with Chinese names. The old native names of places should always be literally preserved. How much more beautiful is the old Franco-Indian name *Stadaconda* than *Quebec* for the scene of death of Wolfe! I should be glad to co-operate or correspond with any interested in this matter, so prominent and important at the present juncture.

From *Nature* **31**, 173, 25 December 1884.

Nuclear physics

Widening scope for new heavy-ion accelerators

from Shigeru Kubono

A NEW generation of heavy-ion accelerators is opening up fresh fields of research in the natural sciences — including astrophysics, solid state physics, medicine and geology — as well as finding more conventional application in nuclear physics. One of their biggest successes so far has been the production of new elements, heavier than ever seen before in nuclear reactions. An element of atomic number (Z)=108 has recently been synthesized at GSI Darmstadt in West Germany following the discovery of $Z=106$ and $Z=109$ elements, and experiments have been planned to search for elements of $Z=110$ and heavier. These and other new findings in nuclear physics, along with applications of heavy-ion beams in other areas, were the theme of a two-part symposium* held in Japan.

Since the Second World War, light-ion and electron beams have been used extensively to investigate nuclear structure and reaction mechanisms. Heavy-ion beams, with energies up to 10 MeV per nucleon, made their appearance in the 1960s. Several new generation machines, now with energies of ten to a few hundred MeV per nucleon, have already been completed — principally at Michigan State University, Caen in France (GANIL), and Darmstadt (GSI) — and several more are on their way, including RIKEN in Japan and at Lanzhou in China.

The new heavy-ion beams have a wide range of application in the natural sciences. They are highly effective in cancer radiotherapy, as shown in a recent joint US-Japan cooperative research project (K. Sakamoto, Tohoku University). The main advantages of such beams over other particle beams are the higher efficiency with which they kill cells, the independence of their cell-killing from the stage of the cell cycle and their selectivity for killing tumour cells rather than normal cells. So far, only the Lawrence Berkeley Laboratory has radiotherapy facilities; experiments with carbon, neon and silicon ions have been tried.

Transfer of energy from heavy ions causes a variety of effects at solid surfaces and interfaces. These are the basis of a number of applications that are likely to be of great commercial value, including the reduction of surface reflectivity in scintillators, improvement of thin film adhesion and the establishment of electrical con-

tacts, and the implantation of ions into semiconductors (T. Tombrello, California Institute of Technology).

Heavy-ion beams can also be used in mass spectrometry, allowing detection of isotope ratios as small as 10^{-14} . In addition to various archaeological applications, this will make it possible to use neutrino detectors such as ^{71}Ge or ^{205}Tl rather than conventionally used ^{37}Cl . These detectors would provide a good sensitivity for neutrinos from the earliest stage of the pp chain in stellar evolution.

Several quite new problems in nuclear physics can also be tackled with the aid of the new accelerators. One proposal (E. Adelberger, University of Washington, Seattle) is to look for charge symmetry breakdown in the very high excitation energy region of nuclei by comparing heavy ion- and light ion-induced reactions. Adelberger also suggests a search for breakdown of the time-reversal symmetry by using the elastic scattering of polarized neutrons from certain heavy nuclei. Other important topics that can now be tackled experimentally are the production of high-density nuclear matter in relativistic heavy-ion scattering (S. Nagamiya, University of Tokyo) and the important role that may be played by very neutron-rich nuclei in the early stage of the Universe (K. Sato, University of Tokyo).

Quantum electrodynamics has been tested by looking at the decay of the vacuum by the spontaneous positron emission in the supercritical fields of a giant nuclear system of high atomic number. In the presence of the very strong electric fields produced by such systems the vacuum is predicted to decay by positron emission. Such a phenomenon has been observed experimentally at GSI and the result shown to be compatible with theory (B. Müller, J. Goethe University, Frankfurt). The phenomenon appears to follow the production of a giant nuclear system of $Z=173-188$.

Conventional nuclear physics has also received a boost from the new heavy-ion accelerators. In addition to the discovery of the $Z=108$ element and plans for the search for even heavier elements and nuclei that are far off the stability line on the nuclear chart (G. Münzerberg and O. Klepper, GSI), giant resonances — particularly high energy octupole resonances — have been shown to be better excited by heavy ions (D. Youngblood, Texas A. & M. University). Some remarkable new detectors were also described. One is a 'crystal ball' that covers almost all the space around the target where the nuclear

reaction takes place with a NaI or BGO crystal. Studies of very high spin states and structure have made tremendous progress with the help of such a detector (J. Garrett, Niels Bohr Institute), as have studies of the decay property of giant resonances (J. Beene, ORNL, USA) and of quasi-molecular resonances (V. Metag, University of Giessen, FRG).

Quasi-molecular states seem to have gained recognition as one of the general features of nuclei. A rotational band based on the quasi-molecular configuration now seems quite likely to exist below the threshold energy (S. Kubono, University of Tokyo) and even in very heavy nuclei (M. Gai, Yale University). A new reaction mechanism has been proposed in which fission does not go through compound nucleus formation; this process becomes more prominent as the incident energy of the heavy-ion beam increases (B. B. Back, Argonne National Laboratory). A time-dependent Hartree-Fock calculation for heavy-ion scattering has been used to predict that two colliding nuclei with low angular momenta will not fuse under some conditions. Possible evidence of such a low angular momentum cut-off has been measured (T. Nomura, RIKEN).

One of the most controversial subjects discussed at the symposia concerned absolute fusion cross-sections below the Coulomb barrier — a subject related to the proton-proton chain in stellar evolution (T. Kajino, Tokyo Metropolitan University). According to some reports, a contribution from quasi-elastic scattering processes is very important in reproducing the absolute values (T. Tamura, University of Texas; C. Signorini, Padova; N. Taki-gawa, Tohoku University); but an alternative explanation involves a process of neck formation (A. Iwamoto, JAERI).

As the incident energy increases, the reaction mechanism of heavy-ion scattering seems to evolve dramatically (C. Détraz, GANIL; R. G. Stokstad, Lawrence Berkeley Laboratory). The cross-sections of fusion reactions decrease in a systematic manner (S. M. Lee, University of Tsukuba; S. Harar, SACLAY) and new reaction mechanisms, such as break-up/fusion and fragmentation processes (H. Utsunomiya, Michigan State University; D. Gross, HMI; E. Rehm, Argonne National Laboratory), and a pre-equilibrium process (C. K. Gelbke, Michigan State University), start to play an important role. Another interesting subject in nucleus-nucleus scattering at an intermediate incident energy is the absolute cross-sections for pion production near the absolute threshold energy. The existing theories underestimate the π^0 production cross-sections by more than an order of magnitude. Why they do so remains an open question. □

*The 1984 INS-ROKEN International Symposium on Heavy Ion Physics was held in two parts: 'New Scope of Heavy Ion Science', Tokyo, 24-25 August, 1984, and 'Heavy Ion Nuclear Physics', Mt Fuji, 27-31 August, 1984. The proceedings will be published in Supplements to the *Journal of the Physical Society of Japan*.

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Epstein-Barr virus and carcinoma

SIR — A News and Views article by Rickinson¹ raises very important questions on the biology of Epstein-Barr virus (EBV). Since our report² that EBV genomes are regularly present in the epithelial cells of nasopharyngeal carcinoma (NPC), the discrepancy between *in vitro* experiments that failed to detect EBV receptors and the *in vivo* findings has been an important point of discussion. Rickinson discusses experiments by Sixby *et al.*^{3,4} on the detection of EBV genomes in exfoliated epithelial cells from throat washings and the finding that EBV can infect epithelial cells only if the virus is derived from throat washings, and not from tissue culture.

The data are interesting but not necessarily convincing. Sixby *et al.* have no evidence that the EBV-containing cells really originate from the throat, and their demonstration of the infection of epithelial cells involves an undefined system: throat washings contain a wide variety of components, including viruses of different kinds. Thus one must question their conclusion that mutants of EBV which are infectious to epithelial cells regularly exist or evolve. This would imply a very efficient and directed mutation of isolates of EBV obtained by collecting cell lines established from umbilical cord blood lymphocytes by immortalization with EBV from throat washings. The observation by Sixby may merely confirm the findings of Kehlifa and Menezes⁵, who were able to infect non-natural target cells of EBV (EBV receptor-free cells) by preinfection or simultaneous infection with Sendai viruses. The samples used by Sixby *et al.* may have contained pseudotypes of EBV particles and other viruses infectious for epithelial cells⁶.

Our recent data, published too late for Rickinson to have been aware of, suggests an alternative interpretation of Sixby's data. We have found that EBV can replicate in the parotid gland of patients lacking antibodies to major EBV-related early antigens. This site of replication allows lifelong production of EBV without effective stimulation of the humoral antibody response because the EBV-producing cells are in the lumen of the salivary duct and not in contact with the bloodstream. Perhaps these cells are shed with the saliva and are then detected in the throat. The virus released may in fact be responsible for a constant supply by new infection of peripheral B lymphocytes with EBV genomes. Rickinson also mentions the production by Yiwan *et al.* of an EBV-producing cell line after fusion of Tupaia cells with lymphoblastoid cells⁸. This may be a model for the establishment of persistent low level lytic cycles of EBV in specific cells unable to suppress EBV replication in the way peripheral lymphocytes from convalescents seemingly do.

Besides lytic infection, EBV is characterized by its ability to undergo

latency. It has long been known that in Burkitt's lymphoma and NPC biopsies, virus particles are only synthesized after one or two days of *in vitro* cultivation. As virus replication invariably leads to the lysis of the host cells, latency is a condition for a potential DNA tumour virus. In the case of NPC, the tumour cells are epithelial. The EBV genomes may enter these cells by mechanisms discussed above. However, mixed infections are very likely to kill the target cells. In addition, cells that can be infected by this mechanism should be located at the outer layers of the body and are therefore likely to be cells that have completed differentiation and are unable to live much longer. Aspirates of cells from the mucosa may contain such cells which may contain viral genomes but will even so undergo lysis⁹.

This does not mean that any transfer of EBV genomes to epithelial cells is an irreversible event leading invariably to the development of NPC. Nevertheless, the partial activation of EBV genes in lymphocytes harbouring otherwise latent EBV genomes and the consequent transfer of EBV genomes via EBV-mediated cell fusion to EBV receptor-negative epithelial cells¹⁰ would be a simple explanation for the presence of EBV genomes in submucosal epithelial cells. This mechanism would also offer a mechanistic explanation for the postulated activity of environmental agents as risk factors for the development of NPC.

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1. Rickinson, A. *Nature* **310**, 99 (1984).
2. Wolf, H. *et al.* *Nature new Biol.* **244**, 245 (1973).
3. Sixby *et al.* *New Engl. J. Med.* **310**, 1225 (1984).
4. Sixby *et al.* *Nature* **306**, 480 (1983).
5. Kehlifa & Menezes in *Nasopharyngeal Carcinoma: Current Concepts* (eds Prasad, U. *et al.*) 245 (1983).
6. Shapiro *et al.* *Biochim. biophys. Acta* **696**, 19 (1981).
7. Wolf *et al.* *J. Virol.* **51**, 795-798 (1984).
8. Yiwan *et al.* *Scient. sin.* **27**, 284 (1984).
9. Desgranges *et al.* *Int. J. Cancer* **29**, 87 (1982).
10. Bayliss & Wolf *Proc. natn. Acad. Sci. U.S.A.* **78**, 7162 (1981).

Succession theory is too reductionist

SIR — Bryan Finegan (*Nature* **312**, 109-114; 1984) is right to point out that the pendulum of succession theory has swung too far from the holist view to the reductionist, and his article is welcome. Any attempt to categorize all pioneer species in terms of *r*-selection would be perverse, bearing in mind that the heterogeneous nature of the environments that pioneers colonize — from open water through various bare rock materials (sand, gravel, lava) to mature soils. The ability of textbooks to quote successively the 'standard succession', which always starts with lichens, and the *r*-selected char-

acteristics of pioneers, has long puzzled me.

I would like to add two points. First, although he sensibly allows the mechanisms of facilitation, tolerance and inhibition to interact and occur throughout succession, there is a real sense in which facilitation is a first requirement.

Many, if not all, primary successions exhibit a qualitative shift at some stage — from aquatic to terrestrial, nitrogen-free to nitrogen-sufficient, very low to adequate water-holding capacity. These shifts determine whether late-successional species can grow in the initial stages — oak trees do not grow in water — and are achieved typically by autogenic change. In other words, facilitation is a widespread event in the early stages of primary succession because: (1) Species vary in their ability to grow in typical primary succession environments; and (2) the act of growing in such environments induces modification (terrestrialization, N and organic matter inputs). After the shift has been achieved, however, other processes may be much more important.

Second, I think that it is important to point out that not all macronutrients are deficient in early primary succession. Because of the lack of mineral N source in most soils, N is almost always limiting but P and K are almost entirely soil-derived. K is always entirely inorganic in soil, and may increase in availability due to inputs (particularly in maritime environments) and decomposition of minerals and decrease due to leaching and particulate loss. The overall trend is typically downwards. P is initially wholly inorganic, but over time declines in absolute amount, due to particulate loss and to a lesser extent leaching, while shifting gradually to organic forms. Most of the organic P cycles very slowly, if at all, and the solution P concentrations decline.

The net result of an increase in N availability and a decrease in P and K supply should produce a brief period, some time after the initiation of a succession, when productivity is maximal. Interestingly, such a pattern is frequently observed, though not usually ascribed to such a cause.

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Disease and the elm decline

SIR — I read the note by Peter Moore¹ on the historical decline of elms with considerable interest. I pointed out elsewhere² that the presence of elm bark beetle (*Scolytus scolytus*) remains in the fossil record does not prove that Dutch elm disease occurred, as the beetle may not always carry the fungal pathogen responsible for the disease. The only effective means of testing the disease hypothesis is to try to grow any

resting spores of *Ceratocystis ulmi* which may be found in fossil lake sediments in culture. Spores of other fungi from sediments as old as the Tertiary have hitherto been cultured successfully^{3,4}, so viability may not be a problem.

As cereal-type pollen has been shown to pre-date the elm decline^{5,6}, the real problem of interest to palaeoecologists now is probably not so much why the elm declined but how to find corroborative evidence for early agriculture. I suggest that a study of grass silica remains (phytoliths) could provide the answer. These may be washed into lakes from cropped areas and become incorporated in the sediments. Recent work from the United States^{7,8} indicates that the careful, detailed study of phytoliths can provide rather more palaeo-environmental information than was once supposed. Some papers on British phytoliths have been published⁹⁻¹¹ and these provide a foundation from which research could start. Additionally, it may be easier to determine phytoliths than pollen using automated methods, as the number of morphological types which occur is that much lower and their overall structures are simpler. It is always possible that identifiable portions of leaf cuticle, and not just phytoliths deriving from cuticles, can be found in fossil contexts and analysis of these might make research easier.

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1. Moore, P.D. *Nature* **312**, 103 (1984).
2. Maloney, B.K. *Circaea* **2**, 91-96 (1984).
3. Sugiyama, J. *J. Fac. Sci. Tokyo Univ. III (Botany)* **9**, 287-311 (1966); 378-405 (1967); **10**, 29-48 (1968).
4. Sugiyama, J. & Goto, S. *J. Fac. Sci. Tokyo Univ. III (Botany)* **10**, 97-116 (1969).
5. Groenman-van Waateringe, W. in *Landscape Archaeology in Ireland* (eds Reeves-Smyth, T. & Hamond, F.) 217-232; B.A.R., *British Series No.* **116** (1983).
6. Edwards, K.J. & Hiron, K.R. *J. Archaeol. Sci.* **11**, 71-80 (1984).
7. Brown, D.A. *J. Archaeol. Sci.* **11**, 345-368 (1984).
8. Piperno, D.R. *Am. Antiquity* **49**, 361-383 (1984).
9. Parry, D.W. & Smithson, F. *Ann. Bot.* **28**, 169-185 (1964).
10. Parry, D.W. & Smithson, F. *Ann. Bot.* **30**, 525-538 (1966).
11. Blackman, E. & Parry, D.W. *Ann. Bot.* **32**, 199-206 (1968).

Protein antigenicity and protein mobility

SIR — In an article in *Nature*, Westhof *et al.*¹ discuss the proposition that the antigenic determinants of proteins could belong to regions which show high mobility. In developing this correlation they have inadvertently overlooked work by ourselves². Using NMR methods which detect relatively slow motions as well as the fast motions seen in crystals, we observed that "the region of cytochromes *c* around Ile-57 is very flexible and it is striking that the best characterized antigenic site involves amino acid residues 58-62" (ref. 3).

We go on to state that for myoglobin and lysozyme, protein flexibility in charged surface segments is also important for antigenicity.

While not wishing to detract from the work of Westhof *et al.*, we think it right that attention should be drawn to this earlier work, since in the Oxford Enzyme Group we are engaged in a very detailed examination of mobility in relation to antigenicity in the cases of both cytochromes *c* and lysozyme. These studies, using both NMR⁴ and crystallographic methods to analyse mobility, show that the idea that such a correlation exists is confirmed but that mobility must be considered in the broadest context of motions on the slow time scale, perhaps even down to seconds, as well as at $<10^{-9}$ s. This means, of course, that the antigenicity need not be due to the ground state conformation or its mobility but can be related to a conformation of slightly higher energy⁵. Such states will be missed unless both crystallographic and NMR methods are used together.

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1. Westhof, E. *et al.* *Nature* **311**, 123-126 (1984).
2. Moore, G.R. & Williams, R.J.P. *Eur. J. Biochem.* **103**, 543-550 (1980).
3. Urbanski, G.J. & Margoliash, E. *J. Immun.* **118**, 1170-1180 (1977).
4. Robinson, M.N., Boswell, A.P., Huang, Z.-X., Eley, C.G.S. & Moore, G.R. *Biochem. J.* **213**, 687-700 (1983).
5. Moore, G.R. & Williams, R.J.P. *Eur. J. Biochem.* **103**, 523-532 (1980).

Doubts over the Colorado Plateau

SIR — I was puzzled by your comment in a News and Views leading article¹ on calderas "Can anyone now doubt that the Colorado Plateau . . . is a magmatic structure of the same kind?" Frankly, yes! I am wondering if it was Dan McKenzie's letter to *Nature*² which triggered your suggestion. If so, I think that you read something into his discussion that he did not mean to be there. He was speculating on the possible effects of injecting very large amounts of magma (complete provinces) into or beneath the crust throughout regions such as the Colorado Plateau, in order to account for their uplift without any internal deformation. Calderas are related to single volcanic centres and, although some of them are uncomfortably large (from the volcanic hazard point of view), they are all an order of magnitude smaller than the sort of multicentre regional phenomena that McKenzie was considering. Similarly, resurgent doming is a phenomenon of individual volcanic centres, rather than entire provinces. It is (as the name implies) a dome-like uplift, which may be related approximately to a point pressure source, as magma reoccupies the reservoir below. In contrast, the Colorado Plateau has risen like a lift floor, with essentially all the deformation around its margins. The Flat Tops area, in north-west Colorado, shows a similar style of uplift on a smaller scale than the plateau;

strata are flat-lying throughout its uplifted area but almost vertical along the monocline at its western margin.

I think that McKenzie's explanation of the plateau's uplift is probably correct. The region escaped the thermo-tectonic events which affected most of the western United States during the Mesozoic. At about 25 Myr, it was penetrated by scattered, explosive, low-volume, alkaline magmatism which was very loosely related to kimberlite. This carried up lithospheric xenoliths of garnet-lherzolite from depths as great as about 140 km (similar to South Africa), and seems to have originated from the top of the asthenosphere³. Subsequently, there have been large amounts of magmatism all around the plateau but virtually none upon it. The surrounding magmatism is compositionally essentially the same as that which characterizes ocean islands, except for local input from crustal fusion in areas of intense volcanic activity. If such liquids come ultimately from the asthenosphere (as must clearly be the case in ocean basins), then the existence and nature of asthenosphere beneath the plateau becomes the vital question. It appears to be both present at >140 km, and seismically normal^{4,5}. Thence, following the McKenzie model, one deduces that abundant magmas have left the asthenosphere beneath the plateau during the late Tertiary but none has reached the surface. The only point of McKenzie's analysis that I doubt concerns heat flow. This seems rather too low on the plateau to accommodate the massive amounts of geologically-recent intra-crustal and Moho-depth magmatism he envisages. Perhaps the uprising liquids consolidated deeper, within the sub-Plateau lithospheric mantle. Here they would ultimately become an episode of "mantle metasomatism", of the type (one of the types) that Hawkesworth and his colleagues study⁶.

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1. Maddox, J. *Nature* **312**, 193 (1984).
2. McKenzie, D. *Nature* **307**, 616 (1984).
3. Smith, D. & Eherenberg, S.N. *Contr. Miner. Petrol.* **86**, 274-285 (1984).
4. Anderson, D.L. & Dziewonski, A.M. *Scient. Am.* **251**, 58-66 (1984).
5. Parker, E.C. *et al.* *Nature* **312**, 354-356 (1984).
6. Hawkesworth, C.J. *et al.* *Nature* **311**, 331-335 (1984).

Mouse Igx sequence elements in *Drosophila* (erratum)

A letter under the above title from P. A. Tsonis appeared in Scientific Correspondence in the 22 November issue (*Nature* **312**, 314; 1984) with the author's name omitted. □

Interstellar scattering in the inner parts of the Galaxy

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A new survey of the galactic plane for sources with size less than 1 arc s at 327 MHz shows that towards the inner parts of the galaxy for galactic latitudes less than 5°, interstellar scattering is much larger than expected from data at higher latitudes. The enhanced scattering varies both with galactic latitude and longitude. A two-component model for the distribution of scattering matter in the Galaxy is proposed to interpret the observations.

THE properties of the ionized component of the interstellar medium can be inferred from their effects on radio waves propagating through the medium. Scattering of radio waves by density inhomogeneities in the interstellar medium produces a number of observable effects, such as angular broadening of extragalactic sources, interstellar scintillation and temporal broadening of pulsar signals. Radio observations of pulsars have provided information on the properties of the local ionized interstellar medium. However, pulsars are not very effective probes for studying the large-scale properties of the interstellar medium because they have relatively low intrinsic luminosities; the estimated distance to the more distant pulsars may not be reliable. Although angular broadening of extragalactic radio sources does not have these limitations, the magnitude of this effect is so small (<1 arc s) that it has only been studied by interplanetary scintillation (IPS) and very long baseline interferometry (VLBI). At metre wavelengths, IPS is very useful for detecting and estimating the angular sizes of compact components in radio sources as all that is required is a large collecting area¹.

The simplicity of the IPS technique compared with VLBI makes it suitable for studies covering a large number of sources; however, the only existing survey² is restricted to the northern sky and hence provides no information on the scattering strength in the inner parts of the Galaxy. Therefore, we have used the Ooty Radio Telescope (ORT)³, which extends south to a declination limit of -35° , to perform at 327 MHz a new IPS survey, which includes the galactic centre. We find that interstellar scattering (ISS) is larger in the inner parts of our Galaxy and infer the variation of scattering strength with distance from the galactic centre.

Observations

We have studied two regions of the sky defined by

(A) $|b| < 10^\circ$ and $-35^\circ < \delta < 0^\circ$ ($-10^\circ < l < 35^\circ$)

(B) $|b| < 10^\circ$ and $5^\circ < \delta < 30^\circ$ ($170^\circ < l < 210^\circ$)

where δ = declination, b = galactic latitude and l = galactic longitude. Catalogues of extragalactic radio sources at low galactic latitudes are confused by strong galactic sources; we have made, therefore, an unbiased IPS survey of all scintillating sources in regions A and B whether or not they are catalogued. We made drift observations when these regions were 10 – 25° from the Sun. With the 12-beam system of the ORT, we could observe a field of 36 sec δ arc min in declination; each part of the sky was in our beam for 8 min. Scintillating sources were detected from analogue records by their characteristic rapid variations. In addition, the mean and root mean square (r.m.s.) of the recorded data were plotted and examined for scintillating sources appearing as regions of enhanced r.m.s. By requiring that the variation of the r.m.s. of any genuine scintillating source should have the characteristic east–west and north–south beam-

shape of the ORT, we discriminate against terrestrial and solar interference and also confusion from the enhanced background temperature in the galactic plane. An IPS survey of the type described here is free from such confusion because the strong galactic sources are extended and therefore do not scintillate; pulsars are the only galactic sources that would be detected. Power spectral analysis of the recorded data enables us to identify pulsars with visible pulses at 327 MHz, but short period heavily scattered pulsars may exist, appearing as continuum sources. We repeated our observations on all regions that showed possible IPS.

Catalogues of small diameter radio sources in galactic centre region A have been made at 408 MHz for $|b| < 3^\circ$ (ref. 4) and $|b| > 3^\circ$ (ref. 5) at a sensitivity of about 0.7 Jy. All the sources for which we have reliable IPS detection could be identified with sources in these catalogues. However, it is possible that some genuine compact sources have been missed because of interference and telescope failures. To minimize this, we made IPS observations of all sources catalogued in ref. 4 and all those with total flux at 408 MHz > 0.8 Jy catalogued in ref. 5. We observed each source for 5–8 min at the optimal distance from the Sun to determine whether or not it showed IPS. However, we did not survey known sources in the anticentre region.

Sources with compact components were observed subsequently for 15 min each on 5–10 different days at different angular distances from the Sun. The equivalent half-power gaussian diameters were determined from the width of the power spectra of the intensity fluctuations, as measured by the second moment of the power spectra⁶. The required estimation of the interplanetary medium scattering parameters was made from repeated observations of the flat spectrum source 1148–001, whose angular size at 408 MHz is less than 0.015 arc s (ref. 7). A mean solar wind velocity of 400 km s^{-1} has been assumed.

For high galactic latitudes and IPS observations near the Sun, the system temperature of the ORT is about 300 K; with a collecting area of $8,000 \text{ m}^2$ and bandwidth of 4 MHz we can detect a source with scintillating flux (ΔS) around 60 mJy in 5 min. The system temperature of the ORT does not increase by more than 4 dB, apart from a $2^\circ \times 2^\circ$ region around the galactic centre, when the antenna is pointed at the galactic plane. In the anticentre direction the increase in system temperature is negligible except in the neighbourhood of the Crab Nebula. To have a uniform sensitivity limit for our entire survey, we have included only those sources which have $\Delta S \geq 0.2$ Jy. This conservative limit allows us to be confident that our survey is complete to this level of flux in region A. The survey of region B is incomplete; in the limited time available we concentrated on low-latitude regions.

Although our survey has a uniform sensitivity limit of $\Delta S = 0.2$ Jy, the sensitivity limit for the flux in the scintillating component (S_s), varies both with the ecliptic latitude and the angular diameter of the source. At 327 MHz, for angular distances

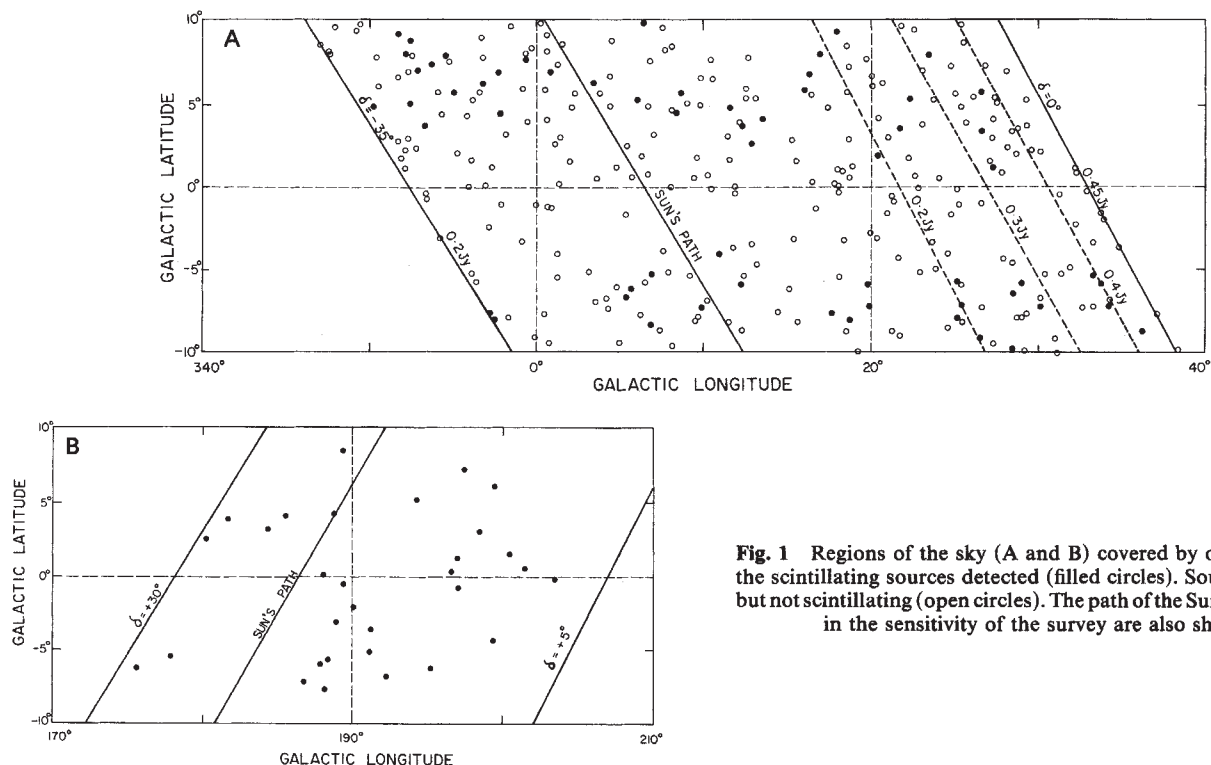


Fig. 1 Regions of the sky (A and B) covered by our survey and the scintillating sources detected (filled circles). Sources observed but not scintillating (open circles). The path of the Sun and variation in the sensitivity of the survey are also shown.

greater than 10° , ΔS of a point source decreases as the angular distance between the Sun and the source increases. As the Sun never comes close to sources at high ecliptic latitudes, the sensitivity of the IPS technique decreases with ecliptic latitude. The maximum absolute value of the ecliptic latitudes of the surveyed regions is about 25° , so that over the surveyed regions the sensitivity to point sources falls at most by 2.5 (ref. 6). Further, because of diameter blurring¹, the sensitivity of the IPS technique decreases as the angular size of the source increases and is too low, at 327 MHz, to be useful for sources with diameter greater than about 0.5 arc s unless the source has a large flux density.

Results

The boundaries of regions A and B and the detected positions of the scintillating sources are shown in Fig. 1. There is a striking difference in the distribution of scintillating sources in the two regions. In the galactic anticentre direction we detected scintillating sources even at $b = 0^\circ$, but in the galactic centre region we did not detect any scintillating source of $|b| < 1.5^\circ$. We divided the latitude range of the regions surveyed into three equal zones, each of width 3.3° (Table 1). Although the number of catalogued sources in the lowest latitude zone in region A was about 60% smaller than in the higher latitude zones because of effects of confusion in the Clark and Crawford⁴ survey, the number of scintillating sources in this zone is six times smaller. In contrast, in the anticentre direction, we detected three times more scintillating sources in the lowest latitude zone even though the survey

was over a smaller area of the sky and is not complete. As the sensitivity of our survey does not depend on galactic latitude, the observed deficiency of sources at low latitudes in region A is a real effect which must be explained.

We next plotted the dependence of the diameter of the scintillating component on galactic latitude for four galactic longitude ranges (Fig. 2). As the number of sources at low latitudes is small, we included four sources which were scintillating, but did not satisfy the criteria set for inclusion in our survey. Two of the sources (1740–261 and 1855–016) have scintillating flux marginally less than the survey limit of 0.2 Jy, whereas the other two (1903+015 and 1915+062) were north of the northern boundary of our survey. In the longitude range $30^\circ > l > -10^\circ$ where the deficiency of scintillating sources for $|b| < 3.3^\circ$ is most pronounced, all six detected scintillating sources in this latitude range have angular diameters larger than 0.4 arc s. As the sensitivity of the IPS technique for sources in this diameter range is less than that for point sources, this argues strongly against the observed deficit of sources at $|b| < 3^\circ$ being an artefact caused by the lower sensitivity of our survey in the galactic plane. The observed large values of the angular sizes of the scintillating sources at low latitudes (Fig. 2a, b) are probably the effects of ISS broadening in these longitude ranges, the deficiency of scintillating sources being a consequence of this broadening. The absence of scintillating sources with $|b| < 1.5^\circ$ requires that the ISS angle at these latitudes is > 1 arc s, consistent with previous observations⁸, at 408 MHz and low galactic latitudes, of enhanced scattering along lines of sight passing through the inner Galaxy.

In the presence of ISS broadening, the observed brightness distribution of a source is the convolution of the true distribution with the point spread function due to the ISM. From our observations alone, we are unable to determine the ISS angle for each source as we do not know the intrinsic sizes of the detected scintillating sources. In principle, one could determine the ISS angle if one had made IPS observations at two frequencies, as the scattering angle increases roughly as the square of the wavelength whereas the intrinsic size of the sources changes more slowly. However, as the intrinsic sizes of the scintillating sources at 327 MHz are likely to be between 0 and 1 arc s (Fig. 2d), the lower envelope of the points in Fig. 2a and b is a statistical measure of the ISS angle in these longitude ranges.

Table 1 Break-up of the number of sources at different galactic latitudes

Latitude range	Number of compact sources detected		Number of catalogued sources studied	
	Galactic centre region A	Anticentre region B*	Region A	Region B
$0^\circ < b \leq 3.3^\circ$	5	17	97	ND
$3.3^\circ < b \leq 6.7^\circ$	31	11	146	ND
$6.7^\circ < b \leq 10^\circ$	30	7	141	ND

* Surveyed areas at different latitudes not equal. ND, not done.

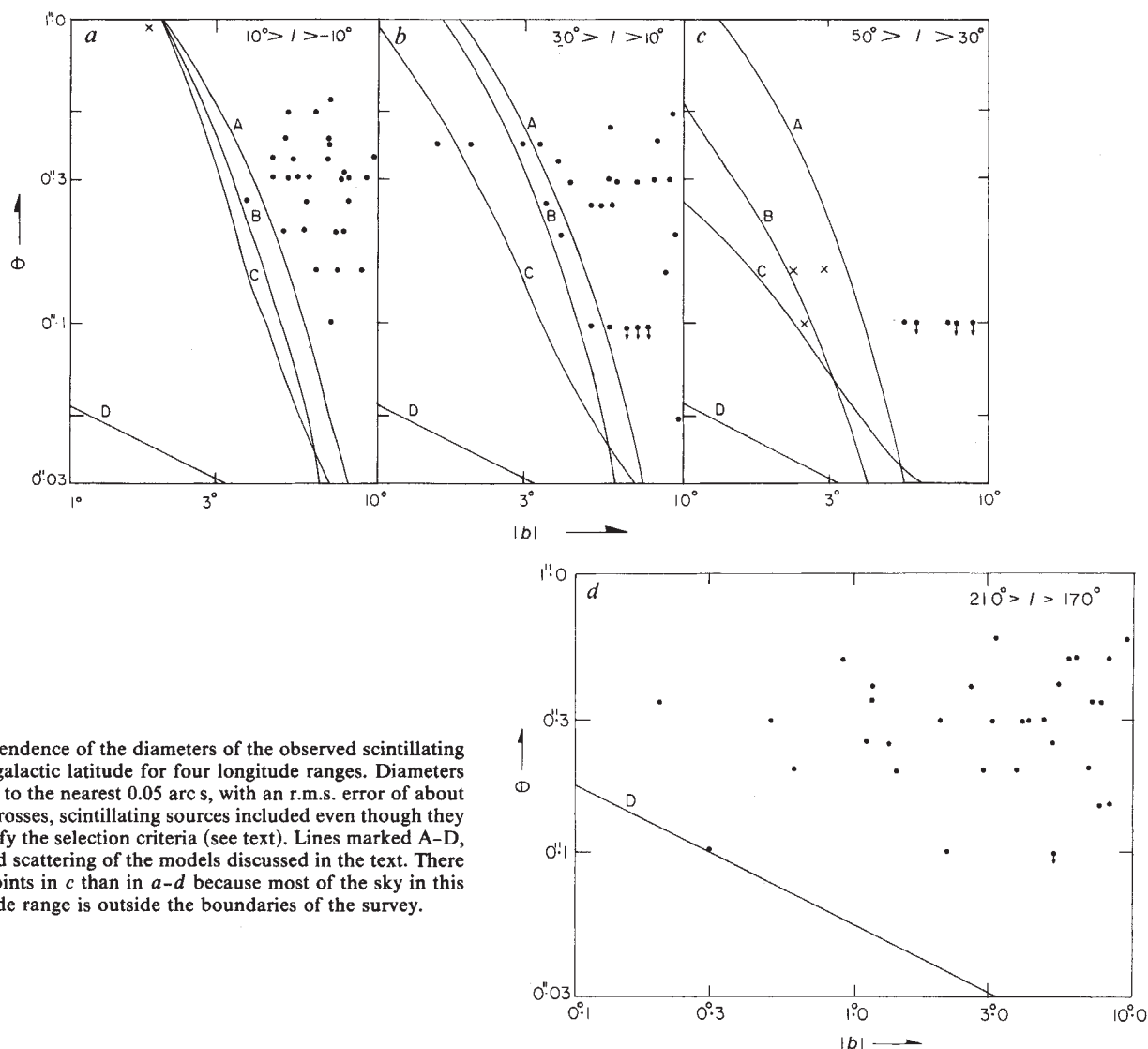


Fig. 2 Dependence of the diameters of the observed scintillating sources on galactic latitude for four longitude ranges. Diameters are rounded to the nearest 0.05 arc s, with an r.m.s. error of about 0.05 arc s. Crosses, scintillating sources included even though they did not satisfy the selection criteria (see text). Lines marked A–D, the predicted scattering of the models discussed in the text. There are fewer points in *c* than in *a*–*d* because most of the sky in this longitude range is outside the boundaries of the survey.

Where scintillating sources are systematically absent, the apparent angular sizes of sources are larger than 1 arc s. The lower envelope of the points in Fig. 2*a* and *b* increases as $|b|$ decreases. The magnitude of ISS broadening varies with both galactic latitude and longitude and its variation with galactic latitude is faster than $(\sin |b|)^{-1/2}$ (line D in Fig. 2). This rules out models of the ISM in which the scattering strength is independent of the galactic radius and the distribution perpendicular to the galactic plane is either uniform or exponential.

Two-component model for scattering

From an analysis of the 80 MHz Cambridge IPS survey supplemented with observations at 151 MHz, it has been concluded that scattering material in the ISM has scale height perpendicular to the galactic plane between 400 and 700 pc (ref. 9). At 80 MHz, for $|b| > 10^\circ$, the ISS angle is given by

$$\theta_{\text{scat}}(\text{arc s}) = 0.12(\sin |b|)^{-1/2} \text{ for } |b| > 10^\circ$$

(ref. 10), after correction for IPS diameters described in ref. 11. As this result is valid only for high galactic latitudes, it is sensitive to the scattering properties of the interstellar medium within about 2 kpc from the Sun. The properties of the local interstellar medium derived from this survey are in general agreement with those estimated from the dispersion and scattering properties of nearby pulsars. The variations of the ISS angle with wavelength depends on the shape of the spectrum of the irregularities responsible for the scattering¹². For a gaussian spectrum the ISS angle increases as $(\text{wavelength})^2$ whereas for a Kolmogorov spectrum it is related to $(\text{wavelength})^{2.2}$. The

extrapolation of the 80 MHz result to $|b| < 10^\circ$ after scaling to 327 MHz as $(\text{wavelength})^2$ giving the largest value for the scattering angle is shown in Fig. 2, line D. At very low galactic latitudes, the ISS angle could be less than this line because of the finite radial extent of our Galaxy. Over most of the sky, the predicted scattering angle is less than or comparable with the resolution limit of the IPS technique at 327 MHz (0.05 arc s), and so our survey should not see this scattering. This is consistent with our observations in the galactic anticentre direction, where we see no deficiency of scintillation sources at low galactic latitudes; within the uncertainty of the measurements there is no obvious lower envelope to the observed angular diameter significantly larger than the resolution limit of the IPS technique. However, in the galactic centre directions at low latitudes, the observed scattering is much larger than predicted. The fact that effects of ISS are seen in our survey at 327 MHz shows that this model for scattering in our Galaxy is inadequate at low latitudes. To explain our observations we need an additional component of the ISM whose scattering strength is both much larger and also confined to the inner parts of the Galaxy.

If the distribution of the strength of scattering in our Galaxy were known, the expected angular broadening for any given line of sight could be calculated easily. However, the inverse problem of determining the distribution of the scattering strength from the measured angular broadening in different directions is not easily solved, especially in our case where the angular broadening is measured only in a few directions. To understand the properties of the scattering region, we have compared our observations with the angular broadening predicted by some

simple models. The limitation of such an approach is that we cannot be sure of the uniqueness of the derived model.

For our calculations we have made the following assumptions: (1) The wave number spectrum of the plasma density fluctuations responsible for the scattering is a power law of the form $C_N^2(\text{wavenumber})^{-11/3}$ so that, following ref. 12, the half-power scattering diameter is given by

$$\theta_{\text{scat}}(\text{arc s}) = 1.72 \times 10^{-2} (\lambda_m)^{-2.2} \left[\int_0^s C_N^2(s') ds' \right]^{0.6}$$

where C_N^2 is related to the magnitude of the density fluctuations, λ_m the wavelength of observation in metres and s' the distance along the line of sight, measured in parsecs.

(2) We ignore any possible clumping of the scattering material and assume it is homogeneously distributed in the ISM.

(3) The Galaxy has cylindrical symmetry about the galactic centre and reflection symmetry about the galactic plane. Thus C_N^2 is only a function of r , the distance from the galactic centre, and $|z|$, the height above the galactic plane. We assume that C_N^2 varies exponentially with z with a scale height z_0 that is independent of r . Thus C_N^2 can be expressed as

$$C_N^2(r, z) = C_N^2(r) \exp(-|z|/z_0)$$

For $C_N^2(r)$ we have assumed three simple models (Table 2).

(4) The Sun is located at $z = 0$ and $r = 10$ kpc.

(5) All the scintillating sources we have seen are extragalactic.

For each of these models, and for a range of r_0 and z_0 , we computed the scattering angle for lines of sight in different directions. We fixed C_0^2 by requiring that all models give 1 arc s scattering at $l = 2^\circ$, $b = 2^\circ$, close to the observed parameters of 1740–261, the scintillating source whose line of sight is most close to the galactic centre. The effect of changing the normalization is to slide the model curves vertically in the plots. The goodness of fit was judged by comparing the predicted scattering angles with the lower envelope of the points in Fig. 2. Models with $z_0 \sim 500$ pc are unable to reproduce the observed variation of scattering angle with galactic latitude and longitude. To explain the sharp decrease of the ISS angle from 1 arc s at $|b| \sim 2^\circ$ to ~ 0.1 arc s at $|b| \sim 5^\circ$ in Fig. 2a, these models require the region of enhanced scattering to be confined to within 3 kpc of the galactic centre; thus, they cannot explain the enhanced scattering seen at longitudes as large as 35° . To explain both these observations, we must decrease z_0 . Because of the uncertainty in the scattering angle for $|b| < 1.5^\circ$, where no scintillating sources were seen, we could not uniquely determine z_0 and r_0 , even for the simple models we have considered. However, as most of the acceptable models had z_0 of 50–100 pc, we have assumed $z_0 = 75$ pc and determined r_0 (Table 2). The predicted variation of the scattering angle with latitude is shown in Fig. 2a–c, lines A–C. (The scattering predicted by the models is outside the range of Fig. 2d, hence there are no lines here.)

The constant scattering model is unable to reproduce the observed decrease in the scattering angle with galactic longitude. From our data alone we are unable to distinguish between the linearly-decreasing and the exponential models. The exponential model produces more scattering along the line to the galactic centre, but even this is less than half the observed scattering angle scaled to 327 MHz (ref. 13). If, ignoring the radial decrease in the scattering strength, we assume that material up to two

scale heights in z (13.5%) contributes to the observed scattering, the absence of any estimate of the scattering angle for $|b| < 2^\circ$ implies that our observations are sensitive only to material within 4 kpc of the Sun. Thus, our data shows that the strength of scattering must decrease with r for $r > 6$ kpc. Measurements of the scattering angle close to $|b| = 0^\circ$ would elucidate the scattering strength closer to the galactic centre.

Discussion

The scattering strength of the second component of the interstellar medium at low galactic latitudes is much larger than previously suggested¹⁰ ($C_N^2 \sim 10^{-4} \text{ m}^{-6.67}$; ref. 12). Pulse broadening and interstellar scintillation observations of pulsars whose distance is greater than 2 kpc show that C_N^2 increases with distance^{12,14}, interpreted as owing to enhanced scattering in a component with $z_0 \sim 100 \text{ pc}$ ^{14,15}. Although the earlier model¹⁰ predicts extremely small values for the ISS angle at cm wavelength, high frequency VLBI observations^{16,17} have shown much larger scattering angles for some sources at low latitudes. As there are only very few such observations, however, they have been explained by the sources lying in directions where the line of sight passes through dense regions of the interstellar medium, such as HII regions. VLBI observations of the galactic centre¹³ show a component whose diameter increases roughly as the square of the wavelength, as expected for ISS. However, the scattering angle is four orders of magnitude larger than predicted previously¹⁰, leading to the suggestion that most of the observed scattering takes place within a few parsecs of the galactic centre itself¹⁸ and not in the general interstellar medium. The angular sizes of hydroxyl radical maser sources is larger for more distant sources¹⁹, suggesting that these sources are affected by general interstellar medium scattering, but that the magnitude of this scattering is much larger than predicted¹⁰. VLBI observations at 408 MHz of a sample of 29 low-latitude compact sources⁸ shows that the ISS angle for lines of sight in the inner Galaxy is greater than 0.3 arc s, whereas in the anti-centre direction it is hardly detectable. In all the above examples, the enhanced scattering is seen when the line of sight passes through the inner Galaxy close to the galactic plane, and thus can be explained by scattering in a component of the type required. Although our estimates of the scattering properties of this component may change with more and better observations, it is clear that any general model for scattering in our Galaxy must have at least two components.

As the plasma density fluctuations responsible for scattering are probably proportional to mean density, the mean plasma density is considerably higher also in the region of enhanced scattering. Analysis of the dispersion measures of pulsars shows that the mean density is larger in the inner parts of our Galaxy^{20,21}. As most of the high dispersion pulsars ($DM > 200 \text{ pc cm}^{-3}$) are seen through the inner Galaxy, the distance derived from the dispersion measures is strongly dependent on reliable estimates of the plasma density in this region. Pulse broadening caused by enhanced scattering in the inner parts of the Galaxy leads to an observational selection effect against short period pulsars which could be important in existing pulsar surveys and affect our understanding of the distribution of pulsars in our Galaxy. Searches for millisecond pulsars in the inner parts of our Galaxy could be limited by this pulse broadening even at high frequencies.

As the presence of HII regions along the line of sight increases considerably the observed scattering²², it has been suggested that the enhanced scattering originates entirely from such regions^{8,14}. We have assumed that the scattering material is uniformly spread through the interstellar medium; by estimating the actual scattering angle for all the detected compact sources and by studying the variation of ISS angle with position in the sky, it will be possible to test this hypothesis. The observed distribution of the HII region²³ and radio recombination lines²⁴ suggest the existence of dense thermal plasma in the inner parts of the Galaxy with z_0 of 50–100 pc. It is interesting to speculate whether the region of enhanced scattering is associated with

Table 2 Models for $C_N^2(r)$ and estimated parameters for $z_0 = 75$ pc

Model	Functional form	$C_0^2(\text{m}^{-6.67})$	$r_0(\text{kpc})$
Constant	$C_N^2 = C_0^2$ if $r < r_0$ $= 0$ if $r > r_0$	2.4	7
Linearly decreasing	$C_N^2(r) = C_0^2(1 - r/r_0)$ if $r < r_0$ $= 0$ if $r > r_0$	8.3	7
Exponential	$C_N^2(r) = C_0^2 \exp(-r/r_0)$	56.7	1

this dense plasma. Although in our models we have assumed that scattering strength decreases monotonically with distance from the galactic centre, the estimated density of the thermal plasma shows a peak 4–6 kpc from the galactic centre. The predicted variation of the ISS angle with longitude for $|b| < 0.5^\circ$ is different for these two cases, we cannot distinguish between

them, as estimates of angular broadening occur only to $|b| \sim 2^\circ$. Extension of our observations down to $b = 0^\circ$, either by making IPS observations at high frequencies or by other techniques, will improve considerably our model for C_N^2 . This may allow us to understand the relation between the density fluctuations and the physical processes in the interstellar medium.

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1. Little, L. T. & Hewish, A. *Mon. Not. R. astr. Soc.* **138**, 393–406 (1968).
2. Readhead, A. C. S. & Hewish, A. *Nature* **236**, 440–443 (1972).
3. Swarup, G. *et al. Nature phys. Sci.* **230**, 185–188 (1971).
4. Clark, D. H. & Crawford, D. F. *Aust. J. Phys.* **27**, 713–727 (1974).
5. Large, M. I. *et al. Mon. Not. R. astr. Soc.* **194**, 693–704 (1981).
6. Rao, A. P., Bhandari, S. M. & Ananthakrishnan, S. *Aust. J. Phys.* **27**, 105–120 (1974).
7. Clarke, R. W. *et al. Mon. Not. R. astr. Soc.* **146**, 381–397 (1969).
8. Dennison, B. *et al. Astr. Astrophys.* **135**, 199–212 (1984).
9. Readhead, A. C. S. & Duffett-Smith, P. J. *Astr. Astrophys.* **42**, 151–153 (1975).
10. Duffett-Smith, P. J. & Readhead, A. C. S. *Mon. Not. R. astr. Soc.* **174**, 7–17 (1976).
11. Readhead, A. C. S., Kemp, M. C. & Hewish, A. *Mon. Not. R. astr. Soc.* **185**, 205–220 (1978).

12. Rickett, B. J. *A. Rev. astr. Astrophys.* **15**, 479–504 (1977).
13. Lo, Y. K. *et al. Astrophys. J.* **249**, 504–512 (1981).
14. Cordes, J. M., Weisberg, J. M. & Boriakoff, V. *Astrophys. J.* (in the press).
15. Hall, A. N. *Mon. Not. R. astr. Soc.* **191**, 751–762 (1980).
16. Geldzahler, B. J. & Schaffer, D. B. *Astr. Astrophys.* **76**, L21–L22 (1979).
17. Geldzahler, B. G. & Schaffer, D. B. *Astrophys. J.* **248**, 132–137 (1981).
18. Backer, D. C. *Astrophys. J.* **222**, L9–L12 (1978).
19. Bower, P. J. *et al. Astrophys. J.* **242**, 1080–1101 (1980).
20. Ables, J. G. & Manchester, R. N. *Astr. Astrophys.* **50**, 177–184 (1976).
21. Harding, D. S. & Harding, A. K. *Astrophys. J.* **257**, 603–611 (1982).
22. Prentice, A. J. R. & ter Haar, D. *Mon. Not. R. astr. Soc.* **146**, 423–444 (1969).
23. Downes, D. *et al. Astr. Astrophys. Suppl.* **40**, 379–394 (1980).
24. Lockman, F. J. *Astrophys. J.* **209**, 429–444 (1976).

Platelet-derived growth factor induces rapid but transient expression of the *c-fos* gene and protein

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Exposure of quiescent mouse fibroblasts to platelet-derived growth factor induces mRNA from the c-fos proto-oncogene within 10 min followed by synthesis of nuclear c-fos proteins. These are amongst the earliest described nuclear events that follow a mitogenic stimulus.

DESPITE an extensive knowledge of the molecular architecture of the proto-oncogenes (*c-onc*), little is known about the functions of their products^{1–3}. The *c-sis* gene codes for a subunit of platelet-derived growth factor (PDGF)^{4,5} and the *c-erb-B* gene may encode the epidermal growth factor (EGF) receptor⁶, suggesting roles for these proto-oncogenes in the regulation of cell growth. We are studying the proto-oncogene *c-fos*, whose viral cognate, *v-fos*, is the transforming gene of two murine retroviruses that cause osteosarcomas with short latency and transform fibroblasts in culture^{7–12}. The *c-fos* gene is conserved among vertebrates^{9,13} and differentially expressed during development¹⁴, suggesting that its product is required for growth or differentiation of certain tissues. The *c-fos*-specific RNA is not expressed in most pre- or postnatal mouse tissues. Expression of a 2.2-kilobase (kb) *c-fos* RNA has been detected, however, in extra-embryonic tissues, adult bone marrow and monocytes^{3,14–17}.

The predicted product of the *c-fos* gene differs from the *v-fos* gene product at its carboxy terminus¹⁰. These differences do not seem to be important for transformation as the *c-fos* gene can transform fibroblasts if first modified in two ways: (1) by the addition of a transcriptional enhancer element and (2) by the replacement of 3'-noncoding sequences with heterologous viral sequences¹⁸. The latter modification is thought to interrupt an interaction between sequences in the 3'-coding and noncoding sequences that may inhibit translation¹⁸. Neither change affects the coding region. Thus, rat 208F fibroblasts can be transformed by a hybrid gene containing *c-fos* promoter and coding sequences linked to viral 3'-untranslated sequences containing an enhancer¹⁸. These cells (R-MMV cells) contain a series of proteins differing in post-translational modification, that are totally encoded by the introduced mouse *c-fos* sequences¹⁹. Both *c-fos* and *v-fos* proteins are nuclear¹⁹.

Another proto-oncogene, *c-myc* (whose product is also a nuclear protein^{20,21}), is expressed transiently when fibroblast cultures are exposed to certain polypeptide mitogens²². We have

therefore examined the expression of the resident *c-fos* gene in normal mouse fibroblasts and find that even though *c-fos* mRNA is barely detectable in quiescent, confluent cultures of NIH 3T3 or BALB/c/3T3 cells, it is rapidly induced following exposure to any of the mitogens PDGF, fibroblast growth factor (FGF) or tetradecanoylphorbol-13-acetate (TPA). Expression of *c-fos* mRNA is maximal at 20 min and decreases thereafter. Synthesis and post-translational modification of *c-fos* proteins are readily detectable within the first 30 min of mitogen stimulation, are maximal by 60 min and then decrease. The rapidity of the response and our detection of *c-fos* mRNA induction even when protein synthesis is inhibited, suggest that this is a primary event in the pleiotropic response to mitogens.

Rapid induction of *c-fos* mRNA

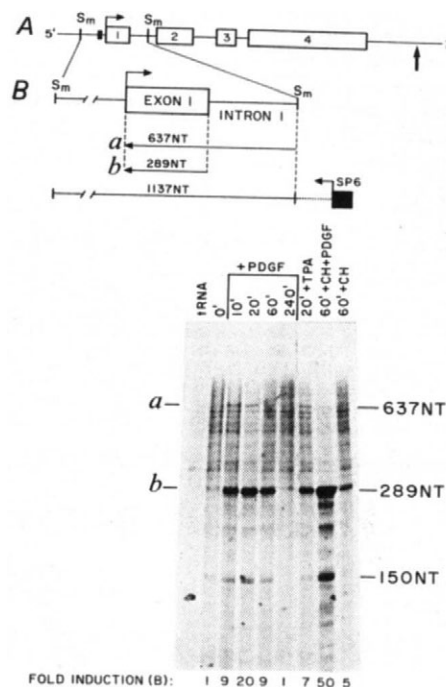
We exposed confluent cultures of NIH 3T3 cells to 0.83 nM PDGF for 4 h in the continuous presence of 35 μ M cycloheximide, which inhibited protein synthesis by >95%. PDGF alone increased the content of *c-fos* RNA threefold, the combination of PDGF and cycloheximide increased the *c-fos* RNA level 10-fold and cycloheximide alone increased the level twofold. Puromycin also augmented the increase in *c-fos* RNA. This suggested that protein synthesis was unnecessary for, and might even inhibit, an increase in *c-fos* RNA content, for example, by attenuation of transcription and/or by stimulating *c-fos* RNA degradation.

Studies on differentiation of monocytic and myelomonocytic cells in response to TPA revealed that, in this system, *c-fos* gene transcription can be activated very rapidly²³. Accordingly, we tested whether brief exposure of resting fibroblast cultures to PDGF would induce *c-fos* RNA. The *c-fos* gene transcripts were characterized by hybridization with ³²P-labelled complementary RNA (cRNA) synthesized using SP6 phage polymerase²⁴, followed by ribonuclease digestion and electrophoresis of the protected cRNA. The cRNA probe was transcribed from a *Sma*I fragment of 1,137 nucleotides encompassing the putative murine

Fig. 1 Analysis of *c-fos* RNA. **A**, Molecular structure of the mouse *c-fos* gene based on nucleotide sequence analysis¹⁰. Expected sizes of the protected *c-fos* mRNA transcripts are indicated, as are the positions of the putative 5' cap ($\cap$), polyadenylation signal ($\uparrow$), TATA box ($\blacksquare$), exon ($\square$), intron ($-$), SP6 phage promoter ($\blacktriangle$) and vector ($\cdots$). **B**, Analysis of *c-fos* transcripts. Total RNA from PDGF-treated cells was used for RNA protection experiments. Times of induction (min) and type of treatment are indicated. As a control for self-hybridization of the probe, one hybridization contained 10 μ g tRNA instead of cellular RNA. Fold induction is indicated at the bottom of the figure. **a**, Expected size 637 nucleotides; **b**, expected size 289 nucleotides. The sizes of the protected fragments were determined relative to denatured ³²P-end-labelled pBR322 *Taq*I fragments as size markers.

Methods: 150-mm dish cultures of NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS). After reaching confluence the medium was replaced with 20 ml of DMEM 0.5% CS for 1 day. Partially purified PDGF (0.4%, from CM-Sephadex³⁴; at this step of the purification, PDGF concentration assayed by mitogenesis equals the concentration estimated by radioreceptor assay³⁰) was dissolved in 1 mM acetic acid and added to a final concentration of 0.83 nM. The same volume of a solution of bovine serum albumin (BSA, 1 mg ml⁻¹) dissolved in 1 mM acetic acid was added to a control dish. Cycloheximide (35 μ M) was added 2 min before PDGF. TPA (250 μ g ml⁻¹) was added from a stock solution in dimethyl sulfoxide (DMSO): the final DMSO concentration was 0.25%. After induction the cells were lysed in 4 M guanidine thiocyanate and total RNA was isolated⁴⁶. RNA was quantitated by measuring A_{260} . A 1,137-bp *Sma*I-*Sma*I fragment, spanning the 5'-untranscribed region and the first exon and part of the first intron of the mouse *c-fos* gene, was cloned in the *Sma*I site of the expression vector pRVII722 (provided by Dr Angerer). To generate run-off transcripts, the plasmid was linearized by *Hind*III, which cuts just downstream of the inserted *c-fos* sequence relative to the site of the SP6 phage promoter in pRVII722. cRNA run-off transcripts were synthesized in a reaction mixture containing 40 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 10 mM dithiothreitol (DTT), 50 U ml⁻¹ RNasin (Promega Biotec), 400 μ M GTP, ATP and CTP, 13 μ M α -³²P-UTP (650 Ci mmol⁻¹; ICN), 1 μ g linearized template and 1,000 U ml⁻¹ SP6 phage polymerase (isolated according to Butler and Chamberlain⁴⁷) for 1 h at 40 °C. Total RNA (10 μ g) was mixed with 5 ng ³²P-labelled cRNA (specific activity 109 d.p.m. μ g⁻¹) in 30 μ l 0.4 M NaCl, 1 mM EDTA, 80% formamide, 40 mM PIPES, pH 6.5. The mixture was heated at 85 °C for 5 min and incubated for 4 h at 37 °C, then for 10 h at 30 °C. After hybridization, 300 μ l of RNase digestion buffer (5 mM EDTA, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5) were added containing 30 μ g ml⁻¹ RNase A and 2 μ g ml⁻¹ RNase T1 and incubated for 30 min at 30 °C. RNase digestion was stopped by adding SDS (final concentration 0.7%) and proteinase K (0.15 μ g ml⁻¹) and incubation was continued for 30 min at 37 °C. The samples were extracted once with phenol/chloroform and ethanol-precipitated with 10 μ g tRNA as carrier and 1.0 M NH₄OOCCH₃. The precipitates were dissolved and re-precipitated with 0.5 M NH₄OOCCH₃. Dried pellets were resuspended in 5 μ l formamide/dye mixture and loaded on a 5% sequencing gel containing 8.3 M urea and 90 mM Tris-borate pH 8.3; 2.5 mM EDTA. The gel was exposed with a fluorescent screen for 3.5 h at -70 °C.

c-fos gene promoter, the first exon and part of the first intron¹⁰ (Fig. 1A). If transcription starts at the presumed 5'-cap site then the primary, unspliced transcripts should protect 637 nucleotides of the probe and the spliced mature mRNA should protect 289 nucleotides of the cRNA from ribonuclease digestion (Fig. 1A). Ribonuclease-resistant hybrids of ~289 nucleotides were formed with RNA extracted from PDGF-treated NIH 3T3 cell cultures (Fig. 1B); this band was undetectable with RNA from untreated cultures. Quantification of this band indicated at least 20-fold induction of *c-fos* mRNA by 20 min exposure of cells to PDGF. Addition of PDGF plus cycloheximide for 60 min resulted in a 50-fold increase in spliced *c-fos* RNA relative to a ninefold increase with PDGF alone. By measuring the radioactivity in the 289-nucleotide fragment, we estimate that after a 20-min exposure to PDGF, 0.0001% of NIH 3T3 cell RNA (0.005% mRNA) is *c-fos* mRNA. Assuming a cellular RNA content of 6 pg, this corresponds to about 5-10 copies of *c-fos* mRNA per



cell after 20 min of induction. Essentially identical kinetics of induction were observed with RNA from stimulated cultures of BALB/c/3T3 cells.

Protection of two other fragments was observed in the cRNA protection experiment (Fig. 1B). One fragment, corresponding in size to that expected for unspliced *c-fos* RNA (**a**), was not detected with RNA from either unstimulated cells or cells treated with PDGF for 4 h. With all other samples the amounts of the ~637-nucleotide fragment were similar. Cycloheximide did not increase the amount of this fragment, suggesting that transcription rate was unaffected. Protection of a cRNA fragment of ~150 nucleotides was also prominent with samples in which the 289-nucleotide fragment was detected (Fig. 1B). The identity of the smaller fragment is unknown but it could represent transcription from alternate promoter sites or splicing within the usual first exon. Alternatively, it could be an artefact of the analysis, as several other bands of varying intensity were also observed.

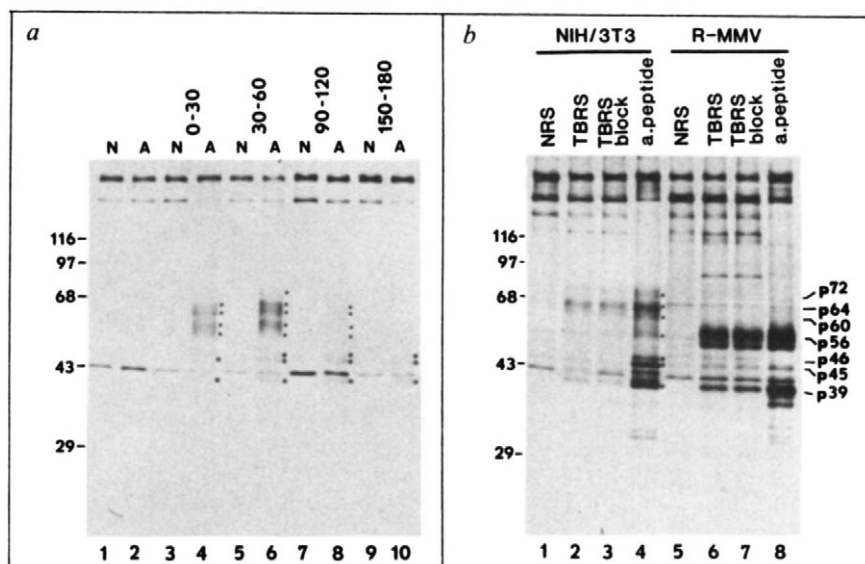
Expression of *c-fos* proteins

We next looked for protein products of the *c-fos* gene by immunoprecipitation of ³⁵S-methionine-labelled cultures using an antiserum directed against a 26-amino acid peptide (M2 peptide, residues 127-152 of the 380-amino acid predicted *c-fos* protein)^{10,25}. BALB/c/3T3 cell cultures were labelled with ³⁵S-methionine for 30 min, starting at various times after addition of 0.67 nM PDGF. Six to eight polypeptides were immunoprecipitable from PDGF-treated but not from control cultures (Fig. 2a). Some of these proteins are *c-fos* gene products and some are unrelated (Fig. 3). Exposure to PDGF for as little as 30 min induced labelling of these immunoprecipitated proteins with hardly any change in the overall rate of protein synthesis. Labelling was maximal by 60 min after addition of PDGF and was decreased markedly 3 h after PDGF addition. The relative incorporation of ³⁵S-methionine into each protein varied with time; the apparent sizes of all the species of relative molecular mass (M_r) between 56,000 (56K) and 72K seemed to increase slightly 30-60 min after PDGF addition. None of these proteins was immunoprecipitated using non-immune rabbit serum (Fig. 2a), nor if excess M2 peptide was used to block specific binding to M2-peptide antiserum (Fig. 4a).

Previous analysis of R-MMV cells has shown that *c-fos* proteins are very heterogeneous due to post-translational modifica-

Fig. 2 Synthesis of *c-fos* proteins in PDGF-treated 3T3 cells. **a**, Time course of *c-fos* protein synthesis; **b**, specificity of immunoprecipitation.

Methods: **a**, BALB/c/3T3 cell cultures were treated with 0.67 nM pure PDGF for 0 (lanes 3, 4), 30 (lanes 5, 6), 90 (lanes 7, 8) or 150 min (lanes 9, 10) before the addition of 100 μ Ci of 35 S-methionine. Another culture received an equivalent volume of BSA in 1 mM acetic acid for 30 min before labelling (lanes 1, 2). After a further 30 min of incubation, cultures were washed, lysed and one-third volumes were immunoprecipitated with 1 μ g IgG equivalent of non-immune rabbit serum (N, lanes 1, 3, 5, 7 and 9) or 1 μ g affinity-purified IgG to M2 peptide (A, lanes 2, 4, 6, 8 and 10). Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis. Proteins related to *c-fos* are marked by a closed square, unrelated proteins by an open circle. A band of M_r 43,000, observed in all samples, is probably actin. **b**, Portions of a NIH 3T3 cell lysate (labelled for 30 min starting 30 min after addition of 0.83 nM pure PDGF) and a R-MMV cell lysate (labelled for 30 min) representing 1/20 of a 35-mm dish culture in each case, were immunoprecipitated with 1 μ l normal rat serum (lanes 1, 5), 1 μ l TBRS (lanes 2, 6), 1 μ l TBRS mixed with 1 μ g M2 peptide (lanes 3, 7) or 0.3 μ g affinity-purified IgG to M2 peptide (lanes 4, 8). Proteins precipitated from PDGF-treated NIH 3T3 cells by antiserum to M2 peptide are indicated at the right and by a closed square (*c-fos*-related proteins) or open circle (proteins not related to *c-fos*) adjacent to lane 4. For labelling conditions see Fig. 3 legend. Except where noted, confluent 35-mm dish cultures of 3T3 cells were incubated in 1 ml DMEM containing 1% normal methionine concentration and 0.5% CS for 40–48 h. Additions of PDGF (essentially homogeneous, purified through phenyl-Sepharose³⁴, dissolved in 1 mM acetic acid containing 1 mg ml⁻¹ BSA), or an equal volume of 1 mg ml⁻¹ BSA in 1 mM acetic acid, and 35 S-methionine (100 μ Ci of $\sim$ 1,000 Ci mmol⁻¹; Amersham/Searle) were made directly to the medium. Cultures were lysed after washing with cold Tris-buffered saline by adding 0.5 ml of RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 100 U ml⁻¹ Trasylol, 10 mM sodium phosphate, pH 7.0) and scraping. Lysates were clarified at 20,000g for 60 min at 4°C. IgG or antisera were added as indicated. After 1 h at 0°C, 1 mg Pansorbin (Calbiochem) was added for 1 h. Immunoprecipitates were centrifuged through a solution of 10% sucrose in RIPA, then washed repeatedly by centrifugation in RIPA⁴⁸. Immunoprecipitations with rat antitumour serum or normal rat serum utilized goat antiserum to rat IgG added 30 min before Pansorbin. Immunoprecipitates were dissociated by incubation at 100°C for 2 min in 2% SDS, 20% 2-mercaptoethanol, 10% glycerol, 0.1 M Tris-HCl, pH 6.8, and one-half of each sample analysed on a SDS-polyacrylamide gel⁴⁹ (12.5% acrylamide/0.10% bis-acrylamide). Gels were stained to visualize marker β -galactosidase, phosphorylase, BSA, ovalbumin and carbonic anhydrase and impregnated with diphenyloxazole (PPO)⁵⁰. Dried gels were exposed to pre-sensitized film at -70°C. Exposure times: **a**, 10 days; **b**, 4 days.



tion, although there are several reasons to suspect that some of the apparently-induced proteins were not products of the *c-fos* gene¹⁹. First, antisera to M2 peptide, or sera from rats with *v-fos*-induced tumours (TBRS), can precipitate also an unrelated cellular protein, p39, from cells expressing either *c-fos* or *v-fos*^{19,25}. This protein appears to be associated with the *fos* gene products^{25,26}. Increased immunoprecipitation of labelled p39 might be expected following PDGF treatment, whether or not its synthesis is induced. Second, one mRNA induced after 3–4 h of exposure of BALB/c/3T3 cells to PDGF (cDNA clone pBC-JB)²⁷ shares a short region of homology with *c-fos*²⁸, hence this induced sequence has been named *r-fos*²⁸. Coincidentally, the M2 peptide encompasses the entire 12-amino acid region of *c-fos*/*r-fos* homology. Thus the antiserum to M2 peptide could potentially recognize a putative *r-fos* protein. (The complete *r-fos* coding sequence has not been determined and the size of its protein product is unknown.)

To elucidate the identities of the immunoprecipitated proteins, NIH 3T3 cells were labelled with 35 S-methionine for 30 min, starting 30 min after addition of 0.83 nM PDGF; lysates were immunoprecipitated with antiserum to M peptide (Fig. 2b, lane 4) or with TBRS (Fig. 2b, lane 2). TBRS specifically precipitated the 56–72K proteins recognized by M2-peptide antiserum but was less efficient at precipitating the presumed p39 proteins and failed to precipitate two proteins of M_r 45K and 46K (p45 and p46). These two proteins were not precipitated in appreciable amounts from R-MMV cells with either TBRS or M2-peptide antiserum (Fig. 2b, lanes 6 and 8). Some of the proteins precipitated with TBRS may be *r-fos* gene products, as in principle this antiserum could recognize the region of homology between *r-fos*, *c-fos* and *v-fos*. Therefore, we tested whether excess M2 peptide could block the precipitation by TBRS of any proteins,

and found that it could not (Fig. 2b, lanes 3 and 7), suggesting that none was recognized solely through the shared peptide region. Furthermore, tryptic peptide analysis confirmed that all four immunoprecipitated proteins of M_r 56–72K were homologous to each other and to two authentic murine *c-fos* gene products synthesized in R-MMV cells (Fig. 3e–h). Thus the different sizes of the *c-fos* products in NIH 3T3 cells and R-MMV cells presumably result from differential post-translational modification. The M_r 39K proteins precipitated from PDGF-stimulated NIH 3T3 mouse cells and from R-MMV rat cells were also highly conserved (Fig. 3a, b). p45 and p46 were very similar and also shared some peptides with p39 (Fig. 3c, d); their genetic origin is unknown.

PDGF and other mitogens

The dependence of p56–72 *c-fos* induction on PDGF concentration was investigated by labelling with 35 S-methionine for 30 min, starting 30 min after PDGF addition. With either NIH 3T3 cells (data not shown) or BALB/c/3T3 cells (Fig. 4a) *c-fos* protein synthesis was maximal at PDGF concentrations that saturate PDGF binding sites at 37°C (1.0 nM)^{29–31} and half-maximal at the concentration for half-maximal binding (0.3–0.5 nM)^{30,31}. These doses are greater than those required for stimulation of DNA synthesis (half-maximal below 0.1 nM; refs 32–34 and data not shown). p56–72 *c-fos* synthesis was detectable at 50% mitogenic doses of PDGF, however (Fig. 4a). The rate of p56–72 *c-fos* synthesis was difficult to quantify because of the number of species involved, but may approach 0.005% of total protein synthesis during the 30-min labelling period. Synthesis of p56–72 *c-fos* was also stimulated by basic pituitary FGF (120 ng ml⁻¹) or TPA (100 μ g ml⁻¹), but was increased only slightly by EGF (8.3 nM; Fig. 4b).

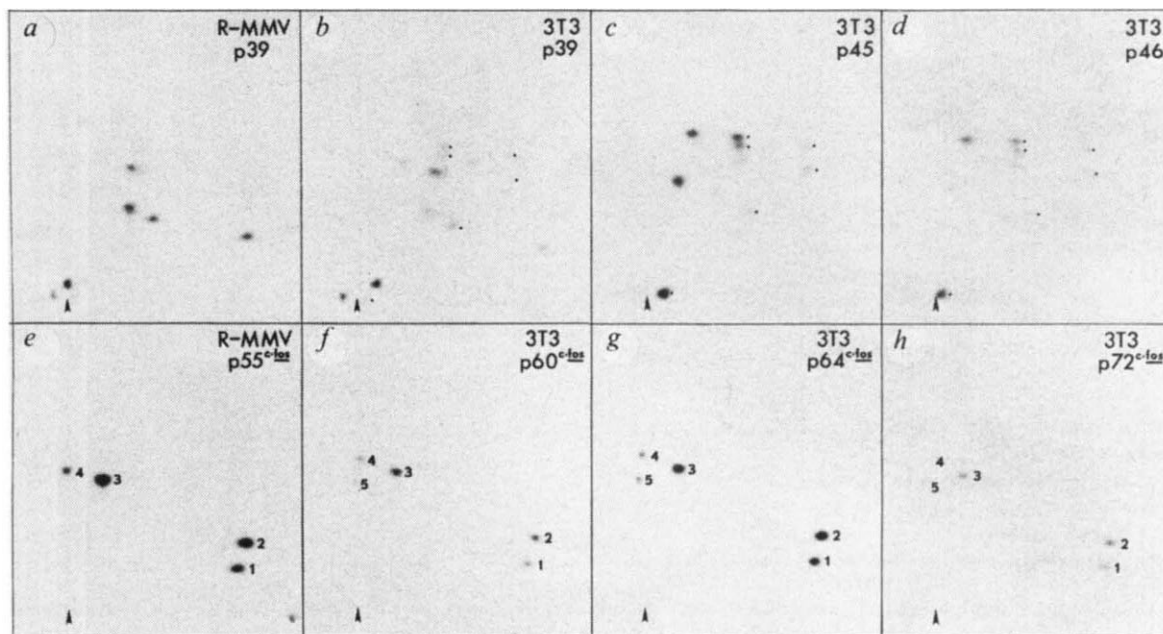
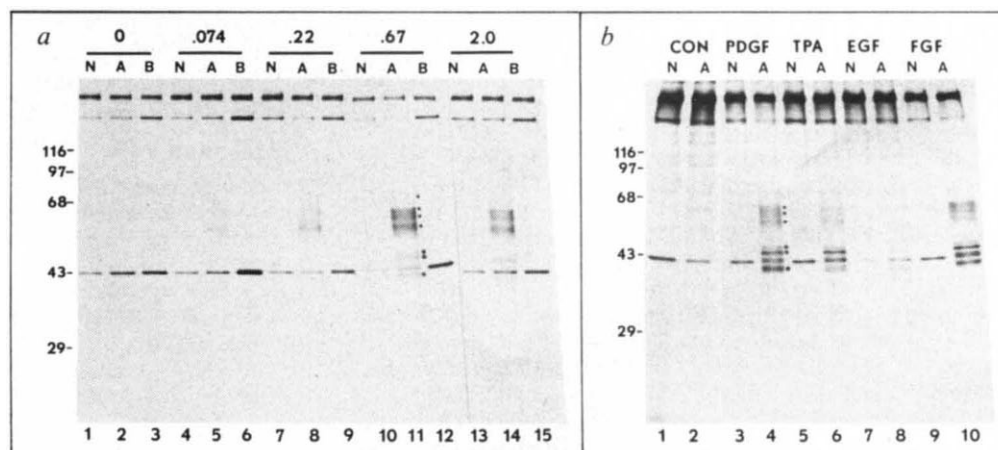


Fig. 3 Tryptic peptide maps of ^{35}S -methionine-labelled proteins immunoprecipitated from PDGF-treated cells. *a*, R-MMV cell p39 (1,720 c.p.m., 4-day exposure); *b*, NIH 3T3 cell p39 (1,100 c.p.m., 4 days); *c*, NIH 3T3 cell p45 (500 c.p.m., 8 days); *d*, NIH 3T3 cell p46 (600 c.p.m., 8 days); *e*, R-MMV cell p55 $^{c-fos}$ (960 c.p.m., 8 days); *f*, NIH 3T3 cell p60 $^{c-fos}$ (700 c.p.m., 8 days); *g*, NIH 3T3 cell p64 $^{c-fos}$ (860 c.p.m., 8 days); *h*, NIH 3T3 cell p72 $^{c-fos}$ (560 c.p.m., 8 days). Peptides in common between murine p39, p45 and p46 are indicated in *b*, *c* and *d* by closed circles (peptide maps of mixtures not shown). Peptides found in murine *c-fos* proteins are numbered 1-5 (*f*, *g*, *h*); all of these except number 5 co-migrate with peptides found in the murine *c-fos*-gene product synthesized in rat R-MMV cells (*e*) (peptide maps of mixtures not shown). From the murine *c-fos* gene sequence¹⁰, we predict that trypsin should generate three methionine-containing peptides small enough to be resolved in our analysis. One is found without mutation in *v-fos* (residues 149-153, MAAAK), one is slightly altered (1-16, single change, S to F) and one is significantly altered in charge (residues 109-113, AGMVK to AEMVK)¹⁰. Analysis of tryptic peptides from p55 $^{v-fos}$ showed the expected co-migrating peptide (number 1), peptides corresponding closely to peptides 3 and 4, and a new peptide more acidic than peptide 2 (data not shown). (Compare with ref. 51; peptide maps shown in inverse orientation.)

Methods: A confluent 35-mm dish culture of NIH 3T3 cells was incubated for 2 days in DMEM containing 1% normal methionine concentration and 0.5% CS. The volume was then reduced to 0.4 ml and 0.83 nM pure PDGF was added for 30 min, followed by 2 mCi ^{35}S -methionine for 30 min. A 35-mm culture of R-MMV cells (clone MMV6B) was incubated for 30 h in DMEM containing 1% normal methionine concentration and 5% dialysed CS before the volume was reduced to 0.4 ml and 1 mCi ^{35}S -methionine added for 30 min. Cultures were washed, lysed and four-fifths of each sample immunoprecipitated with 3 μg affinity-purified IgG to M2 peptide (see Fig. 2). Analytical immunoprecipitations of the remainder of each lysate are shown in Fig. 2b. The polypeptides indicated (Fig. 2b) were excised from the dried gel, eluted, oxidized with performic acid and digested with trypsin as described elsewhere⁵². Half of each digest was analysed by electrophoresis at pH 4.72 (anode at left, origin arrowed) and ascending TLC⁵³. Chromatograms were dipped in molten 2-methylnaphthalene containing 0.4% PPO and exposed to pre-sensitized film at -70°C .

Fig. 4 Induction of *c-fos* proteins by different concentrations of PDGF and other mitogens. *a*, Dose-dependence of *c-fos* protein synthesis; *b*, effects of other mitogens on *c-fos* protein synthesis.

Methods: *a*, BALB/c/3T3 cells were exposed to various concentrations of pure PDGF, diluted in 1 mM acetic acid containing 1 mg ml⁻¹ BSA, for 30 min before the addition of ^{35}S -methionine for 30 min. The final concentrations of PDGF were 0 (lanes 1-3), 0.074 nM (lanes 4-6), 0.22 nM (lanes 7-9), 0.67 nM (lanes 10-12) or 2.0 nM (lanes 13-15). Each lysate was immunoprecipitated with non-immune rabbit serum (N, lanes 1, 4, 7, 10 and 13), 1 μg affinity-purified IgG to M2 peptide (A, lanes 2, 5, 8, 11 and 14) or 1 μg IgG to M2 peptide preincubated with 3 μg M2 peptide (B, lanes 3, 6, 9, 12 and 15). All the samples were run on the same gel and exposed for 10 days, but lanes 10-12 and 13-15 have been rearranged in the figure. *b*, NIH 3T3 cells were exposed to BSA (lanes 1, 2), pure PDGF (2.5 nM, lanes 3, 4), TPA (0.1 mg ml⁻¹, lanes 5, 6), EGF (8.3 nM, lanes 7, 8) or FGF⁵⁴ (0.12 μg ml⁻¹, lanes 9, 10) for 30 min before the addition of ^{35}S -methionine for 30 min. Each sample was immunoprecipitated with non-immune rabbit serum (N, lanes 1, 3, 5, 7 and 9) or affinity-purified IgG to M2 peptide (A, lanes 2, 4, 6, 8 and 10). All samples were run on the same gel and exposed for 5 days.



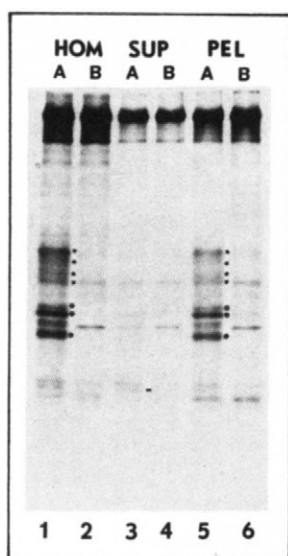


Fig. 5 Subcellular localization of induced *c-fos* proteins. A 35-mm dish of NIH 3T3 cells was treated with partially purified PDGF (0.83 nM) in 0.4 ml medium for 30 min before the addition of 300 μ Ci 35 S-methionine for 30 min. After washing with cold saline the cells were scraped from the dish in 0.4 ml of 1 mM DTT, 10 U ml $^{-1}$ Trasylol, 10 mM HEPES, pH 7.4 and homogenized in a Teflon/glass homogenizer. Half of the sample was removed and the remainder centrifuged at 600g for 5 min at 4 °C. One volume of twice-concentrated RIPA buffer (see below) was added to each fraction. Following centrifugation, small aliquots of each fraction were analysed by SDS-polyacrylamide gel electrophoresis. Certain proteins were found in the supernatant but not in the pellet fraction, suggesting that contamination of the latter fraction with cytoplasm or unbroken cells was negligible (data not shown). Half of the remainder of each fraction—total homogenate (lanes 1, 2), supernatant (lanes 3, 4) and pellet (lanes 5, 6) was then immunoprecipitated with either 1 μ g affinity-purified IgG to M2 peptide (A, lanes 1, 3 and 5) or 1 μ g IgG to M peptide preincubated with 3 μ g M peptide (B, lanes 2, 4 and 6). The gel was exposed for 2 days.

Localization of induced *c-fos* proteins

Immunofluorescence and cell fractionation methods have shown that *c-fos* proteins synthesized in R-MMV cells are nuclear¹⁹. Fractionation of PDGF-treated NIH 3T3 cells by homogenization and low-speed centrifugation followed by immunoprecipitation, suggests that *c-fos* proteins in these cells are nuclear also (Fig. 5). All proteins specifically immunoprecipitated with antiserum to M2 peptide, including p56-72 *c-fos*, p39, p45 and p46 were found in the low-speed pellet fraction containing nuclei (Fig. 5). The induced *c-fos* proteins have not yet been detected by immunofluorescence.

Discussion

Within 10 min of treatment with 0.83 nM PDGF, a nine-fold induction of *c-fos*-specific mRNA is observed in NIH 3T3 cells (Fig. 1) and BALB/c/3T3 cells (data not shown). Exposure of BALB/c/3T3 cells to PDGF also induces *c-myc* RNA²². In our experiments, *c-myc* mRNA continues to accumulate for at least 4 h after PDGF stimulation of NIH 3T3 cells, whereas *c-fos* RNA is expressed only transiently. In other cell types, there is very rapid induction of the growth hormone gene by steroids and of the prolactin gene by EGF^{35,36}, but mRNA levels do not decrease for several hours.

In the presence of protein synthesis inhibitors, at least 50-fold induction of mature *c-fos* mRNA was detected in NIH 3T3 cells (Fig. 1) and BALB/c/3T3 cells (data not shown). Thus, like *c-myc*, *r-fos* and several other genes activated by PDGF, the *c-fos* gene is 'superinduced' when protein synthesis is blocked by cycloheximide^{22,28}. Cycloheximide may either cause stabilization of *c-fos* mRNA or enhance its *de novo* synthesis, for example through inhibition of the synthesis of a labile repressor

of *c-fos* gene transcription. Although the later possibility has been suggested to explain superinduction of *c-myc* gene²², our experiments support the former possibility for *c-fos*, as the amount of unspliced *c-fos* transcript was not increased in conditions of superinduction.

The synthesis of *c-fos* proteins is induced rapidly by PDGF (Fig. 2a), thus PDGF binding²⁹⁻³¹, signal relay to the nucleus, gene transcription, mRNA processing and transport and protein synthesis all must occur in 30 min. Clearly, the mechanism of signal transduction is very rapid, and may well be a direct response to PDGF binding. Indeed, both the concentration dependence and kinetics of *c-fos* gene activation correlate well with previous estimates of occupation of cell-surface PDGF receptors on NIH 3T3 cells at 37 °C (ref. 31). The kinetics of the decline in *c-fos* mRNA synthesis coincide with internalization and degradation of bound PDGF^{29,30}. PDGF has many rapid effects on resting fibroblasts³⁷⁻³⁹. The transcription of *c-fos* must be induced by an effect of PDGF not detected with EGF. Potentially, the protein-tyrosine kinase activity of the PDGF receptor may have a different specificity from that of the EGF receptor, although the substrate proteins identified to date are common to both factors^{31,40}.

The *c-fos* and *v-fos* proteins are modified extensively after translation. In PDGF-treated NIH 3T3 or BALB/c/3T3 cells, at least four *c-fos*-encoded products could be detected (Figs 2, 3). There may be further modified *c-fos* proteins that are too heterogeneous to be detected or that may not be recognized by the peptide antiserum used in our studies. All four *c-fos* species (p56-72 *c-fos*) and the label in p39 disappeared equally rapidly with a half-life of ~2 h (data not shown). If, as we think, p39 is precipitated because of its association with *c-fos* proteins, then *c-fos* proteins are degraded rather than converted into more heavily modified forms. We estimate that the proteins accumulate to much less than 0.0005% of cell protein (~25,000 molecules per cell), but if only one of the many modified forms of p56-72 *c-fos* is active, then even smaller amounts of active gene product may be present. A small number of other mRNAs, including *c-myc* and *r-fos*, are induced by PDGF^{22,27,28}, although they may not be induced as rapidly as *c-fos*. One protein, CPI⁴¹, whose gene is unknown, is synthesized very rapidly after PDGF addition⁴¹, and is nuclear⁴², similar to products of *c-fos* and *c-myc*.

What is the biological significance of rapid *c-fos* RNA and protein synthesis in response to PDGF, FGF and TPA? Studies with BALB/c/3T3 cells have allowed the classification of mitogens as 'competence' or 'progression' factors⁴³ which cooperate for a full mitogenic response. The former category includes PDGF, FGF and TPA; the latter EGF and the insulin-like growth factors⁴³. This classification is not as clear in some other cell types, however, and it is interesting that we could observe induction of *c-fos* proteins with both NIH 3T3 and BALB/c/3T3 cells. The products of the *c-fos*, *c-myc* and other induced genes might all cooperate in some common cellular response or might each mediate a different biological response. Their nuclear localization suggests that they may affect nuclear functions, such as the activation of other genes. In the case of *c-myc*, fibroblasts constitutively synthesizing *c-myc* RNA have a decreased requirement for PDGF for growth⁴⁴. Perhaps constitutive synthesis of *c-fos* RNA and proteins in R-MMV cells also contributes a relaxation of competence factor requirements for cell growth.

Rapid induction of *c-fos* transcripts in PDGF-treated BALB/c/3T3 cells has been reported recently⁴⁵.

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1. Bishop, J. M. *A. Rev. Biochem.* **52**, 301-354 (1983).
2. Hunter, T. *Scient. Am.* **251**, 70-79 (1984).
3. Müller, R. & Verma, I. M. *Curr. Topics Microbiol. Immunol.* **112**, 73-116 (1984).
4. Doolittle, R. F. *et al. Science* **221**, 275-277 (1983).
5. Waterfield, M. D. *et al. Nature* **304**, 35-39 (1983).
6. Downward, J. *et al. Nature* **307**, 521-527 (1984).
7. Finkel, M. P., Biskis, B. O. & Jinkins, P. B. *Science* **151**, 698-701 (1966).
8. Finkel, M. P., Reilly, C. A. Jr, Biskis, B. O. & Greco, I. L. *Proc. Symp. Colston Res. Soc.* **24**, 353-366 (1973).
9. Curran, T., Peters, G., Van Beveren, C., Teich, N. & Verma, I. M. *J. Virol.* **44**, 674-682 (1982).
10. Van Beveren, C., van Straaten, F., Curran, T., Müller, R. & Verma, I. M. *Cell* **32**, 1241-1255 (1983).
11. Curran, T. & Verma, I. M. *Virology* **135**, 218-228 (1984).
12. Van Beveren, C., Enami, S., Curran, T. & Verma, I. M. *Virology* **135**, 229-243 (1984).
13. van Straaten, R., Müller, R., Curran, T., Van Beveren, C. & Verma, I. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3183-3187 (1983).
14. Müller, R., Slamon, D. J., Tremblay, J. M., Cline, M. J. & Verma, I. M. *Nature* **299**, 640-644 (1982).
15. Müller, R., Tremblay, J. M., Adamson, E. D. & Verma, I. M. *Nature* **304**, 454-456 (1983).
16. Gonda, T. J. & Metcalf, D. *Nature* **310**, 249-251 (1984).
17. Müller, R., Müller, D. & Guilbert, R. *EMBO J.* **3**, 1887-1890 (1984).
18. Miller, A. D., Curran, T. & Verma, I. M. *Cell* **36**, 51-60 (1984).
19. Curran, T., Miller, A. D., Zokas, L. M. & Verma, I. M. *Cell* **36**, 259-268 (1984).
20. Donner, P., Greiser-Wilke, I. & Moelling, K. *Nature* **296**, 262-266 (1982).
21. Hann, S. R., Abrams, H. D., Rohrschneider, L. R. & Eisenman, R. N. *Cell* **34**, 789-798 (1983).
22. Kelly, K., Cochran, B. H., Stiles, C. D. & Leder, P. *Cell* **35**, 603-610 (1983).
23. Mitchell, R., Zokas, L., Schreiber, R. D. & Verma, I. M. *Cell* (in the press).
24. Green, M. R., Maniatis, T. & Melton, D. A. *Cell* **32**, 681-694 (1983).
25. Curran, T., Van Beveren, C., Ling, N. & Verma, I. M. *Molec. cell. Biol.* (in the press).
26. Verma, I. M. *et al. Cancer Cells: Growth Factors and Transformation* (Cold Spring Harbor Laboratory, New York, in the press).
27. Cochran, B. H., Reffel, A. C. & Stiles, C. D. *Cell* **33**, 939-947 (1983).
28. Cochran, B., Zullo, J., Verma, I. M. & Stiles, C. D. *Science* (in the press).
29. Heldin, C.-H., Westermark, B. & Wasteson, A. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3664-3668 (1981).
30. Bowen-Pope, D. F. & Ross, R. *J. biol. Chem.* **257**, 5161-5171 (1982).
31. Cooper, J. A., Bowen-Pope, D. F., Raines, E., Ross, R. & Hunter, T. *Cell* **31**, 263-273 (1982).
32. Antoniadis, H. N., Scher, C. D. & Stiles, C. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1809-1813 (1979).
33. Heldin, C.-H., Westermark, B. & Wasteson, A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3722-3726 (1979).
34. Raines, E. W. & Ross, R. *J. biol. Chem.* **257**, 5154-5160 (1982).
35. Murdoch, G. H., Potter, E., Nicolaisen, A. K., Evans, R. N. & Rosenfeld, M. G. *Nature* **300**, 192-194 (1982).
36. Evans, R. M., Birnberg, N. C. & Rosenfeld, M. G. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7659-7663 (1982).
37. Stiles, C. D. *Cell* **33**, 653-655 (1983).
38. Cassell, D., Rothenberg, P., Zhuang, Y.-X., Deuel, T. F. & Glaser, L. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6224-6228 (1983).
39. Habenicht, A. *et al. J. biol. Chem.* **256**, 12329-12335 (1981).
40. Nakamura, K. D., Martinez, R. & Weber, M. J. *Molec. cell. Biol.* **3**, 380-389 (1983).
41. Pledger, W. J., Hart, C. A., Locatelli, K. L. & Scher, C. D. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4358-4362 (1981).
42. Olashaw, N. E. & Pledger, W. J. *Nature* **306**, 272-274 (1983).
43. Scher, C. D., Shepard, R. C., Antoniadis, H. N. & Stiles, C. D. *Biochim. biophys. Acta* **560**, 217-241 (1979).
44. Armelin, H. A. *et al. Nature* **310**, 655-660 (1984).
45. Greenberg, M. E. & Ziff, E. B. *Nature* **311**, 433-437 (1984).
46. Chirgwin, J. M., Przybala, A. E., MacDonald, R. Y. & Rutter, W. J. *Biochemistry* **18**, 5294-5299 (1977).
47. Butler, E. T. & Chamberlain, M. J. *J. biol. Chem.* **257**, 5772-5778 (1982).
48. Sefton, B. M., Beemon, K. & Hunter, T. *J. Virol.* **38**, 957-971 (1978).
49. Laemmli, U. K. *Nature* **227**, 680-685 (1970).
50. Bonner, W. M. & Laskey, R. A. *Eur. J. Biochem.* **46**, 83-88 (1974).
51. Curran, T. & Teich, N. M. *J. Virol.* **42**, 114-122 (1982).
52. Beemon, K. & Hunter, T. *J. Virol.* **28**, 551-566 (1978).
53. Gibson, W. *Virology* **62**, 319-336 (1974).
54. Gospodarowicz, D., Bialecki, H. & Greenberg, G. *J. biol. Chem.* **253**, 3736-3743 (1978).

Induction of *c-fos* gene and protein by growth factors precedes activation of *c-myc*

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Stimulation of fibroblasts with serum or purified growth factors leads to a dramatic induction of expression of both c-fos mRNA and protein within a few minutes, followed by activation of c-myc. This suggests that c-fos induction is a primary event and the earliest known effect on gene expression by growth factors.

RECENT evidence supports the hypothesis that cellular proto-oncogenes (*c-onc* genes) have key roles in the biochemical pathways controlling cell proliferation. Two distinct proto-oncogenes (*c-sis* and *c-erb-B*) control critical events in the transduction of extracellular growth signals into the cell. The *c-sis* gene probably encodes the platelet-derived growth factor (PDGF)^{1,2} and the *c-erb-B* gene product the epidermal growth factor (EGF) receptor³. For *c-erb-B*, the conversion of a cellular growth-factor receptor to an oncogene (*v-erb-B*) has been accomplished by loss of sequences in the regulatory ligand-binding domain³. This could lead to an unregulated receptor which is constitutively expressed in the activated state. For *c-sis*, the exact mechanism by which a normal growth factor-encoding gene has been converted into an oncogene remains unknown.

Further evidence implicates the *c-myc* gene in the control of cell proliferation; it is activated rapidly after stimulation of haematopoietic cells and fibroblasts by mitogens, PDGF^{4,5} and regenerating liver tissue⁶. Moreover, enhanced expression of *c-myc* can eliminate partly the PDGF requirement for cell growth⁷. Nuclear proteins such as *myc*, which are activated by growth factor stimulation, may function as transducing signals to effect the cellular response to growth factors. Here we report that quiescent murine fibroblasts respond to stimulation with serum or individual growth factors by rapidly synthesizing *c-fos* mRNA and protein; this is followed by the appearance of *c-myc*

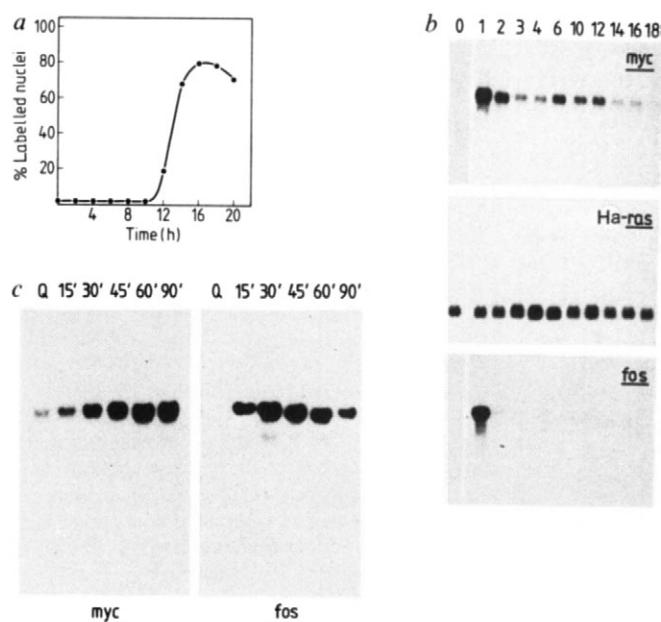
mRNA and the subsequent rapid degradation of almost all *c-fos* mRNA.

Induction of *c-onc* genes

To investigate the involvement of *c-onc* genes in the control of cellular proliferation, we analysed their expression following serum stimulation of quiescent NIH 3T3 cells (clone 7, from D. Lowy) stimulated after 3 days of serum deprivation with 20% fetal calf serum (FCS). This led to synchronous cell cycle initiation (Fig. 1a); the levels of *c-Ha-ras* mRNA (Fig. 1b) and *c-Ki-ras* mRNA (data not shown) increased no more than threefold in serum-stimulated cells at any of the time points analysed. Expression of *c-myc*, however, was induced more than 20-fold 1 h after stimulation with serum (Fig. 1b) followed by a slow decrease until reaching the basal level of quiescent cells after ~18 h (Fig. 1b). The change in *c-fos* expression was even more dramatic, increasing by approximately two orders of magnitude 1 h after serum stimulation and decreasing to basal levels 1 h later (Fig. 1b). Subsequent analyses showed that *c-myc* transcripts appeared at significantly elevated levels after 30 min of stimulation and reached a maximum after 60 min. Induction of *c-fos* clearly preceded the activation of *c-myc* mRNA expression (Fig. 1c) and was detectable as early as 5 min after stimulation (data not shown). It should be noted, however, that the type of RNA analysis used here measures steady-state levels,

Fig. 1 Time course of *c-myc* mRNA expression in quiescent NIH 3T3 cells stimulated with FCS. **a**, Thymidine labelling index following FCS stimulation. The number of labelled nuclei is plotted against time after addition of medium supplemented with 10% FCS. **b**, Time course of *c-myc*, *c-Ha-ras* and *c-fos* expression following FCS stimulation. Sizes of mRNAs are 2.3 kilobases (kb) (*c-myc*), 1.4 kb (*Ha-ras*) and 2.2 kb (*c-fos*) in this and all subsequent figures; 4 μ g poly(A⁺) RNA were analysed per lane. **c**, Time course of *c-myc* and *c-fos* expression following FCS stimulation; 15 μ g total RNA were applied to each lane.

Methods: **a**, NIH 3T3 cells were plated in 35-mm dishes containing 3 ml of medium supplemented with 10% FCS. When the cells were ~70% confluent, the medium was replaced with Dulbecco's modified Eagle's medium (DMEM) containing 0.5% FCS. After 2–3 days, cells were stimulated by adding medium supplemented with 20% FCS. Cells were labelled at 2-h intervals for 2 h with 1 μ Ci ³H-thymidine ml⁻¹. About 500 nuclei were counted in duplicate in each case. **b**, RNA was isolated from cells grown in 600-cm² dishes, selected for poly(A⁺) RNA, separated on formaldehyde-agarose gels and analysed by Northern blotting as described elsewhere^{15,21}. The oncogene-specific probes used were *v-fos* (1.1-kb *Pst*I–*Bgl*II fragment)²², *v-Ha-ras* (BS9, obtained from R. Ellis)²³ and mouse *c-myc* (provided by M. Cole)²⁴.



so we cannot distinguish whether the dramatic increase in the level of *c-fos* mRNA and its rapid disappearance are the result only of a modulated rate of transcription or whether it reflects also modulation of *c-fos* mRNA stability. Transcripts from four other proto-oncogenes tested were either not detectable (*c-sis* and *c-fms*) or did not show changes in the level of expression (*c-abl* and *c-ras*) (data not shown).

Induction by growth factors

Serum contains many growth factors, hormones and other growth-supporting components. We therefore analysed the induction of *c-fos* and *c-myc* expression by the purified growth factors EGF, fibroblast growth factor (FGF) and PDGF, each of which can induce a new round of DNA synthesis when added to quiescent NIH 3T3 cells in serum-free medium. Both FGF and PDGF are efficient in 80–90% of the cells, whereas EGF stimulation leads to cell-cycle initiation in only 50% of the cells (Fig. 2a).

The strong initial induction of *c-myc* expression by FCS was reproduced using either EGF, FGF or PDGF alone (Fig. 2b). Expression of *c-myc*, however, was considerably more stable in FGF- or FCS-treated cells than in EGF- or PDGF-stimulated cells, where the concentration of *c-myc* mRNA decreased to

relatively low levels 2 h after stimulation. The expression of *c-fos* dramatically increased 1 h after stimulation of quiescent NIH 3T3 cells with FGF or PDGF and was then abolished rapidly (similar to FCS-stimulated cells) (Fig. 2b). In contrast, EGF-treated cells showed a considerably lower induction of *c-fos* mRNA expression (Fig. 2b), possibly reflecting the weaker mitogenic effect of EGF on NIH 3T3 cells compared with FGF or PDGF (Fig. 2a). In contrast to *c-myc* and *c-fos*, no changes in *c-Ha-ras* expression were observed after stimulation with EGF, FGF or PDGF (Fig. 2b). These results clearly show that the expression of *c-myc* and *c-fos* in NIH 3T3 cells is rapidly and specifically inducible by purified growth factors. However, a crude preparation of FGF was used in this study, and thus may contain impurities that could enhance its effect on NIH 3T3 cells. Induction of a *c-fos*-related gene (*r-fos*) by PDGF has been observed also (C. Stiles, personal communication).

Superinduction by cycloheximide

Cultivation of quiescent NIH 3T3 cells (in 0.5% FCS) for 4 h with the protein synthesis inhibitor cycloheximide resulted in a slightly increased expression of both *c-myc* and *c-fos* mRNA (Fig. 3). Moreover, stimulation of quiescent NIH 3T3 cells for 4 h with 20% FCS and cycloheximide led to the accumulation

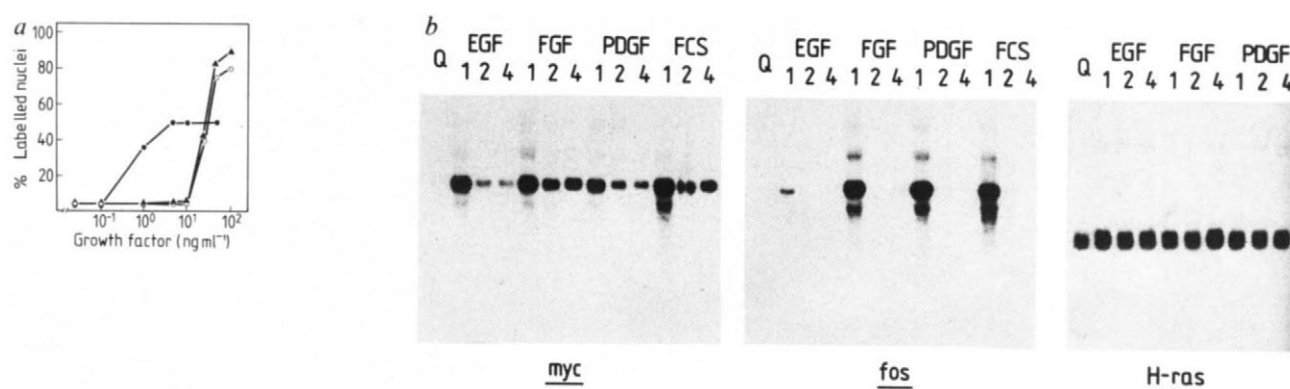


Fig. 2 Induction of *c-myc* and *c-fos* mRNA expression by growth factors. **a**, Thymidine labelling index of NIH 3T3 cells stimulated for 24 h by growth factors. **b**, Northern blot analysis of *c-myc*, *c-fos* and *c-Ha-ras* expression in NIH 3T3 cells in a quiescent growth state (Q), and 1, 2 and 4 h after growth-factor stimulation; 15 μ g total RNZ applied to each lane.

Methods: **a**, Quiescent cells (see Fig. 1 legend) were stimulated with DMEM containing the indicated doses of either EGF (●), FGF (○) or PDGF (△) and labelled for 24 h with 1 μ Ci ml⁻¹ of ³H-thymidine before being processed for autoradiography. **b**, Quiescent cells stimulated with DMEM containing either EGF (10 ng ml⁻¹; BRL), FGF (100 ng ml⁻¹; BRL) or PDGF (100 ng ml⁻¹; BRL) (similar results were obtained with highly purified PDGF), for different periods as indicated.

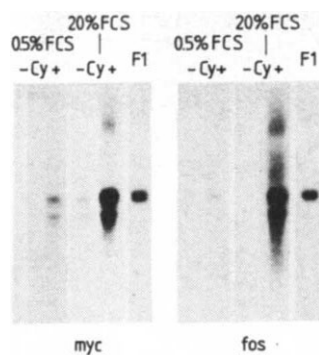


Fig. 3 Superinduction of *c-myc* and *c-fos* mRNA expression. Quiescent NIH 3T3 cells (see Fig. 1 legend) were cultured for 4 h in either 0.5% or 20% FCS with (+Cy) or without (–Cy) cycloheximide ($5 \mu\text{g ml}^{-1}$). F1, NIH 3T3 cells 1 h after stimulation with serum; 15 μg total RNA were analysed as described in Fig. 1 legend. Left-hand lanes (0.5% FCS) were exposed for 15 h, right-hand lanes (20% FCS) for 2 h.

of *c-myc* and *c-fos* RNA to concentrations several fold higher than the maximum observed in the absence of cycloheximide (Fig. 3, lane F1). Hence, *c-myc* and *c-fos* genes may be modulated (repressed) by an unstable protein or by autoregulation and *c-fos* mRNA seems to be considerably more stable in the presence of cycloheximide, presumably because of the absence of a protein (or proteins) that would normally cause rapid degradation following the peak level of expression 1 h after stimulation. Superinduction of *c-myc* expression by cycloheximide has been observed previously in other cells⁴ and in regenerating liver tissue⁶.

Increased expression in proliferating cells

The rapid induction of *c-fos* and *c-myc* expression after growth-factor stimulation suggests that these proto-oncogenes have a primary role in the control of cell proliferation. We therefore examined their expression in rapidly proliferating and quiescent cells. We found increased expression of both *c-myc* and *c-fos* mRNA in growing relative to quiescent cells; the highest concentration of expression was detected in transformed cells (Fig. 4). Thus, there is a correlation between the expression of *c-fos* and *c-myc* proto-oncogenes and cellular proliferative activity. Although expression of *c-myc* in transformed cells approached that in serum-stimulated (1 h) NIH 3T3 cells, the level of *c-fos* mRNA was considerably lower in the transformed cells than in stimulated normal cells (data not shown).

Synthesis of *c-fos* protein

As growth-factor stimulation of NIH 3T3 cells leads to a dramatic induction of *c-fos* mRNA, we investigated *c-fos* protein synthesis in these conditions. Synthesis of the *fos* gene product was measured by immunoprecipitation in both normal mouse fibroblast (NIH 3T3) and normal rat fibroblast (208F)⁸ cell lines. Almost undetectable levels of *c-fos* protein occurred in serum-deprived cells, but 1 h after stimulation we found a dramatic induction of a highly modified relative molecular mass (M_r) 62,000 form of the *c-fos* protein. At 2 h post-stimulation, *fos* protein synthesis was already reduced ~fivefold, the time course of *fos* protein synthesis (Fig. 5a) reflecting the previously observed pattern of mRNA induction (Fig. 1b). In addition to the *fos* protein, p39, the cellular protein complexed to *fos*^{9,10}, was also detected.

In agreement with our RNA data, *c-fos* protein induction by FGF and PDGF was equivalent, whereas induction by EGF

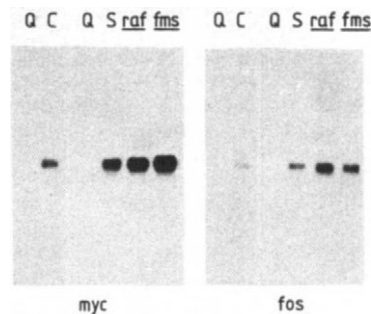


Fig. 4 Increased levels of *c-myc* and *c-fos* mRNA in growing versus quiescent cells. Quiescent NIH 3T3 cells (Q) were compared with normally proliferating non-synchronized NIH 3T3 cells growing in 10% FCS (C); 15 μg total RNA were analysed in this experiment. Expression of *c-myc* and *c-fos* was also analysed in quiescent NIH 3T3 cells (Q) relative to spontaneously transformed NIH 3T3 cells (S) or NIH 3T3 cells transformed by either long terminal repeat-activated *c-raf* gene (*raf*)²⁵ or the McDonough strain of feline sarcoma virus (*fms*)²⁶; 4 μg poly(A⁺) RNA were applied to each lane. Equal amounts of RNA were loaded as determined by ethidium bromide staining of the gel. Northern blot analyses were carried out as described in Fig. 1 legend.

was marginal. However, 10% FCs or the combination of EGF, PDGF and FGF gave stronger stimulation than any of the growth factors alone (Fig. 5b). Using antibodies directed against *myc*-specific synthetic peptides (from Dr Richard Chizzonite), we observed induction also of the *c-myc* protein in NIH 3T3 cells 1 h after stimulation by 10% FCS, FGF, PDGF but not by EGF (data not shown). However, the degree of induction and the amount of *c-myc* protein expressed at this time point was less than that observed with *c-fos*.

Modification of *c-fos* protein

In cells transformed by a *c-fos*-expressing plasmid, the *c-fos* product appears primarily as a M_r 55,000 protein on SDS-polyacrylamide gels during a 20 min pulse-labelling period. This protein is converted to short-lived high M_r forms of 57,000, 60,000 and 62,000 over 2 h (ref. 11). In normal mouse amniotic cells, which express constitutively a high level of *c-fos*, these highly-modified forms appear to be more stable¹¹. In serum-stimulated normal fibroblasts, the majority of the *c-fos* protein was in a highly modified form even in a 20 min pulse-labelling period (Fig. 5a, b). Pulse-chase analyses showed that the small amounts of M_r 55,000 and 57,000 forms detectable after a 15 min labelling period were converted rapidly to the larger forms in 15–30 min of subsequent chase. The profile of *c-fos* protein expression in NIH 3T3 cells and 208F cells was identical. During the 2-day serum starvation period, NIH 3T3 cells become quiescent whereas 208F cells still show a thymidine-labelling index of 10% (data not shown). Thus, *c-fos* induction is not dependent on cells being in the quiescent state. Modification of the *c-fos* protein continued during the chase periods and a stable mature form of M_r 62,000 was generated (p62^{c-fos}), in marked contrast to the pattern of *c-fos* protein modification detected in transformed cells. In these cells the M_r 62,000 form is detected only after 2 h of chase and is very short-lived¹¹.

Immunofluorescence studies

The relatively stable nature of the induced *c-fos* protein indicates that it should be detectable by immunofluorescence staining, which allows a more accurate examination of the time course of appearance of *c-fos* protein as cells are rapidly fixed at each time point. One hour after serum stimulation, high concentrations of *c-fos* protein appeared in the nucleus of NIH 3T3 cells (Fig. 6a). A granular staining pattern occurred throughout the

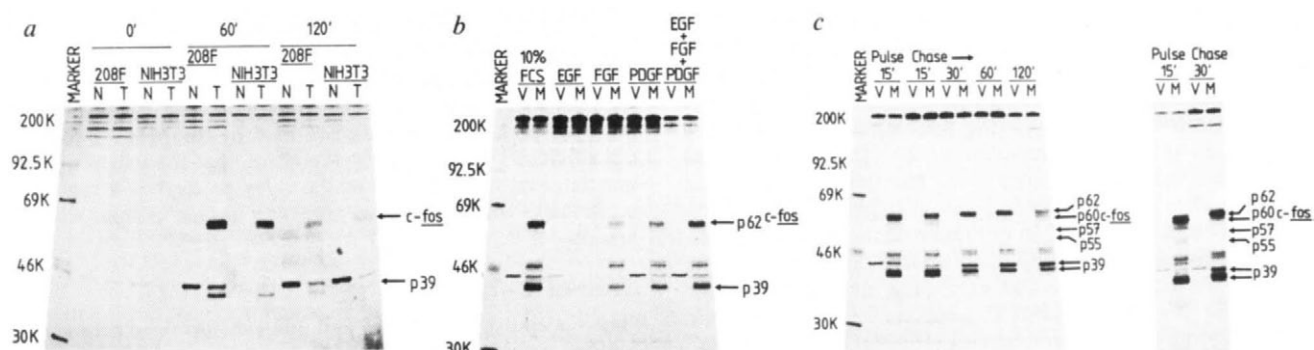


Fig. 5 Induction of *c-fos* protein synthesis by serum and growth factors. **a**, Normal 208F rat fibroblast cells and NIH 3T3 mouse fibroblast cells were stimulated for 0, 1 and 2 h with 10% FCS. Cultures were labelled with ^{35}S -methionine for 20 min at each time point. Cell extracts were prepared and immunoprecipitated with tumour-bearing rat serum (T) and with normal rat serum as control (N). Samples were analysed by electrophoresis on SDS-polyacrylamide gels. The numbers on the left indicate the molecular weights of the ^{14}C -methylated marker proteins (Amersham). For 208F cells each lane represents the immunoprecipitate from 1×10^7 c.p.m. of trichloroacetic acid (TCA)-insoluble protein and for NIH 3T3 cells, the immunoprecipitate from 5×10^6 c.p.m. of TCA-insoluble protein. **b**, NIH 3T3 cells stimulated for 1 h with 10% FCS, 50 ng ml^{-1} EGF, 50 ng ml^{-1} FGF and 50 ng ml^{-1} PDGF were labelled for 15 min with ^{35}S -methionine. Extracts were prepared and immunoprecipitated with V (*v-fos* protein-specific) and M (*fes* protein-specific) peptide antisera. Each lane represents the immunoprecipitate from 10^6 c.p.m. of TCA-insoluble protein. **c**, Pulse-chase analysis of *fes* protein synthesis in NIH 3T3 and 208F cells. Cultures were stimulated for 1 h with 10% FCS, then labelled for 15 min with ^{35}S -methionine. Following the labelling period, medium was removed and replaced with DMEM supplemented with 10% FCS, and cultures were incubated further. NIH 3T3 cell extracts were prepared after the 15-min labelling period and at 15, 30, 60 and 120 min of chase. 208F cell extracts were prepared after the 15-min labelling period and after 30 min of chase. Extracts were immunoprecipitated with affinity-purified *fes*-specific peptide antiserum (M), and as control an affinity-purified peptide antiserum specific for the *v-fos* protein which does not recognize the *c-fos* gene product (V).

Methods: Cell culture was done as described in Fig. 1 legend. For ^{35}S -methionine incorporation, cells were preincubated in 1 ml of methionine-free medium for 20 min before the labelling period. For serum stimulation, this medium was supplemented with 10% dialysed calf serum (Gibco); for growth-factor stimulation the medium was supplemented with the appropriate amount of the relevant growth factor. ^{35}S -methionine labelling was performed by adding $250 \mu\text{Ci } ^{35}\text{S}$ -methionine ($\sim 800 \mu\text{Ci mmol}^{-1}$; Amersham). Preparation of cell extracts, immunoprecipitation and polyacrylamide gel electrophoresis were as described previously¹¹. Characterization of tumour-bearing rat sera as well as the V and M *fes* peptide antisera have been described elsewhere^{10,27}.

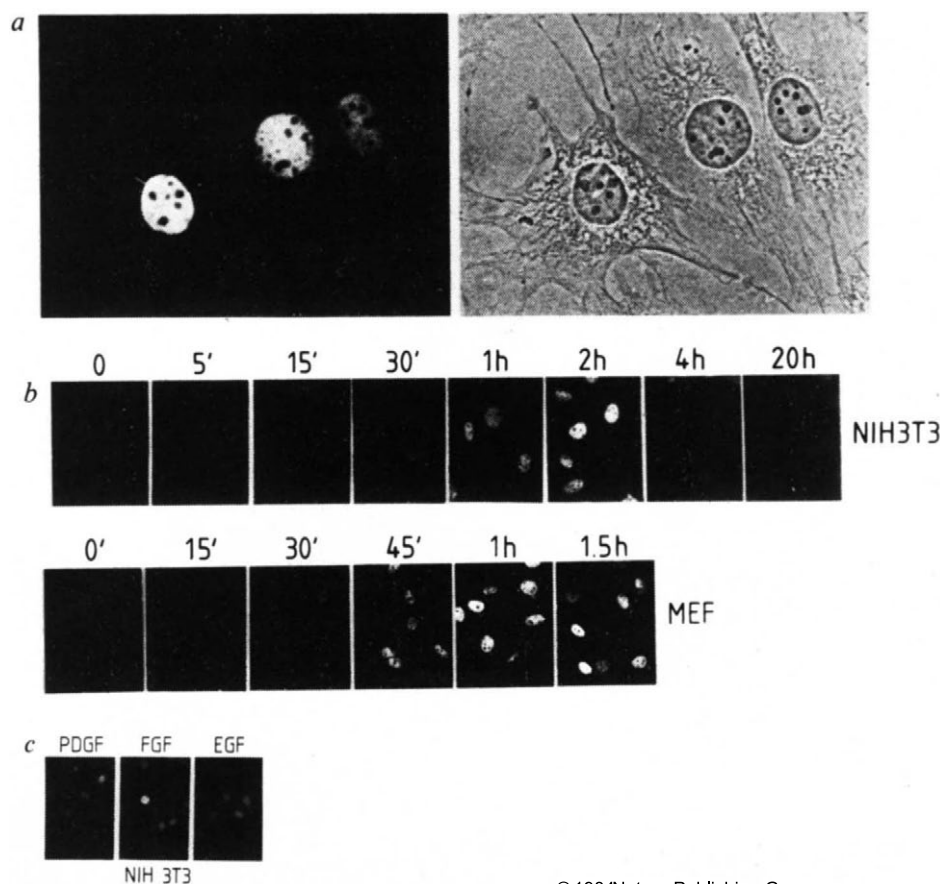


Fig. 6 Detection of *fes* protein induction by immunofluorescence staining. **a**, NIH 3T3 cells were stimulated for 2 h with 10% FCS (see Fig. 1 legend). Cells were prepared for immunofluorescence as described previously¹¹ and stained with TBRS followed by rhodamine-conjugated rabbit anti-rat IgG as the second antibody. Cells were photographed through a $\times 63$ lens (Leitz). Left panel, fluorescence; right panel, phase-contrast. **b**, Time course of *fes* protein induction analysed by indirect immunofluorescence. NIH 3T3 cells were induced by 10% FCS and cultures prepared for immunofluorescence at 0, 5, 15 and 30 min and 1, 2, 4 and 20 h post-stimulation. Tertiary MEFs were also stimulated with serum and prepared for immunofluorescence at 0, 15, 30, 45, 60 and 90 min; antisera were the same as in a. **c**, NIH 3T3 cells induced for 1 h with 50 ng ml^{-1} each of PDGF, FGF and EGF were stained for *fes* expression as described for a. Cell culture was as described in Fig. 1 legend. Cells were fixed with 3% paraformaldehyde and permeabilized with 1% Triton X-100 as described previously¹¹. TBRS and rhodamine-conjugated rabbit anti-rat IgG (Cappel) were used at 1:100 dilutions as described elsewhere¹¹.

nucleus, except the nucleoli. A similar time course of *c-fos* protein induction was observed with NIH 3T3 cells, 208F cells and tertiary mouse embryo fibroblasts (MEFs) (Fig. 6b; data on 208F cells not shown). A small amount of *c-fos* protein was detected in cell nuclei in 5 min (Fig. 6b). Maximal fluorescence was observed at 1–2 h post-induction; it was drastically reduced in ~4 h and undetectable after 20 h. The demonstration that MEF cells also activate *c-fos* expression following stimulation shows that induction is not an artefact of established cell lines.

Immunofluorescence staining of NIH 3T3 cells following induction by EGF, PDGF and FGF (Fig. 6c) confirmed the conclusions from the RNA analyses (Fig. 2b) and immunoprecipitation experiment (Fig. 5b) that PDGF and FGF were more efficient than EGF at stimulating *c-fos* expression (Fig. 5c). Similar observations were made when growth factor-stimulated MEFs were analysed by immunofluorescence (data not shown). In general, stimulated cells gave the same overall appearance of fairly uniform staining independent of the agent used.

Cell proliferation and *c-onc* expression

The very rapid appearance of *c-fos* mRNA and protein after growth factor stimulation argues strongly that *c-fos* activation affects gene expression by primary growth factor, whereas activation of *c-myc* may be a later event. The sudden disappearance of *c-fos* mRNA also contrasts strikingly with the gradual decline of *c-myc* mRNA. The superinduction of both mRNAs by cycloheximide suggests that the levels of *c-fos* and *c-myc* mRNA are in part regulated by the action of a newly-synthesized gene product. Previously, it has been observed that the 3'-untranslated region of the *c-fos* gene is involved in inhibition or expression of the regulation of *c-fos* mRNA stability¹². It is possible that these sequences of the *c-fos* gene or its mRNA are recognized by the regulatory proteins.

Serum induction of fibroblasts results in the presence of high levels of *c-fos* protein for at least 2 h (Fig. 6b). If such *c-fos* stimulation is necessary for the normal cell cycle, a small population of cells which express a high concentration of the *c-fos* protein would be expected, but this is not the case (T.C., unpublished data). Such high expression does not seem to be a normal condition of proliferating fibroblasts in cell culture, but rather is a direct consequence of the growth factor-receptor interaction. Experiments are in progress to determine whether *c-fos* expression is involved in the transition from the G₀ to G₁ phase of the cell cycle rather than in the induction of DNA synthesis.

Implications for transformation

Induction of *c-fos* results in high concentrations of *c-fos* protein expression equivalent to that observed in *c-fos*- or *v-fos*-transformed cells for a period of 2–4 h, but the induced cells maintain a normal morphological phenotype. Thus, *c-fos* protein must be expressed for an extended period of time or even constitutively (such as in virus-infected cells) to induce transformation. Alternatively, as the induced *fos* protein is immediately exten-

sively modified, modified *c-fos* protein may not be capable of inducing transformation. The deletion in the *v-fos* gene seems to prevent modification¹¹, the p75^{gag-*fos-fox*} (ref. 13) transforming protein of FBR-MSV is not modified, and modification of the *c-fos* protein in cells transformed by the MMV plasmid is not efficient¹¹. It is possible, therefore, that the extensive post-translational modification of the *c-fos* product is a regulatory mechanism which affects its ability to induce cellular transformation.

Role of *c-fos*

Previous analyses of tissues and non-synchronized primary cell cultures have shown that *c-fos* is expressed at high concentrations specifically in cells constituting the fetal membranes and placenta¹⁴, bone marrow¹⁵, early fetal liver^{15,16} and differentiated macrophages^{15,16}. The observed stage-specific expression¹⁴ as well as studies using *in vitro* differentiation systems^{15,16} suggest a correlation between *c-fos* expression and differentiation. The expression of exogenous *c-fos* genes can trigger the differentiation of F9 teratocarcinoma stem cells¹⁷. Together, these findings suggest strongly that *c-fos* is involved in the induction of differentiation in particular cell types. In such cells, *c-fos* expression is indeed subject to a different mode of regulation compared with growth factor-stimulated fibroblasts. Thus, non-synchronized primary cultures of amniotic cells show a high concentration of expression in all the cells¹⁵. In addition, expression of *c-fos* in CSF-1 (colony-stimulating factor-1) stimulated macrophages shows a different pattern of cell cycle-related regulation and much less pronounced variations in the level of expression compared with fibroblasts (R.M. and L. Guilbert, unpublished results). This suggests that *c-fos*-expressing cells can be divided into at least two categories: (1) cells (for example, fibroblasts) which show a stringent control of *c-fos* expression and in which *c-fos* may have a key role in the control of cell proliferation and (2) cells (for example, amnion cells, macrophages) which express *c-fos* more or less constitutively and in which *c-fos* has a function in differentiation. The function of the *c-fos* gene product may be associated with the transduction of nuclear signals which, depending on the differentiated state of the cell, may affect regulatory pathways either involved in the control of proliferation or in the induction (or progression) of differentiation. In this context it is interesting to note that EGF has previously been reported to induce the expression of differentiation markers in certain cell types^{18,19}. These observations raise the intriguing question as to how the molecular mechanisms governing cellular proliferation and differentiation may be related to each other.

After submission of this manuscript, another study²⁰ was published reporting rapid induction of the *c-fos* gene following growth-factor stimulation of BALB/c/3T3 cells.

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- Waterfield, M. D. *et al.* *Nature* **304**, 35–39 (1983).
- Doolittle, R. F. *et al.* *Science* **221**, 275–277 (1983).
- Downard, J. *et al.* *Nature* **307**, 521–527 (1984).
- Kelly, K. *et al.* *Cell* **35**, 603–610 (1983).
- Campisi, J. *et al.* *Cell* **36**, 241–247 (1984).
- Makino, H., Hayashi, K. & Sugimura, T. *Nature* **310**, 697–698 (1984).
- Armelin, H. A. *et al.* *Nature* **310**, 655–660 (1984).
- Quade, K. *Virology* **98**, 461–465 (1979).
- Curran, T. & Teich, N. M. *Virology* **116**, 221–235 (1982).
- Curran, T., Van Beveren, C., Ling, N. & Verma, I. M. *Molec. cell. Biol.* (in the press).
- Curran, T., Miller, A. D., Zokas, L. & Verma, I. M. *Cell* **36**, 259–268 (1984).
- Miller, A. D., Curran, T. & Verma, I. M. *Cell* **36**, 51–60 (1984).
- Curran, T. & Verma, I. M. *Virology* **135**, 218–228 (1984).

- Müller, R., Verma, I. M. & Adamson, E. D. *EMBO J.* **2**, 679–684 (1983).
- Müller, R., Müller, D. & Guilbert, L. *EMBO J.* **3**, 1887–1890 (1984).
- Gonda, T. J. & Metcalf, D. *Nature* **210**, 249–251 (1984).
- Müller, R. & Wagner, E. F. *Nature* **311**, 438–442 (1984).
- Johnson, L. K., Baxter, J. D., Vlodavsky, I. & Gospodarowicz, D. *Proc. natn. Acad. Sci. U.S.A.* **77**, 394–398 (1980).
- Benveniste, R. *et al.* *Clin. Endocr. Metab.* **46**, 169–179 (1978).
- Greenberg, M. E. & Ziff, E. B. *Nature* **311**, 433–438 (1984).
- Müller, R. *et al.* *Molec. cell. Biol.* **3**, 1062–1069 (1983).
- Curran, T. *et al.* *J. Virol.* **44**, 674–682 (1982).
- Ellis, R. *et al.* *J. Virol.* **36**, 408–420 (1980).
- Shen-Ong, G. L. C., Keath, E. J., Piccoli, S. P. & Cole, M. D. *Cell* **31**, 443–452 (1982).
- Müller, R. & Müller, D. *EMBO J.* **3**, 1121–1127 (1984).
- Müller, R., Tremblay, J. M., Adamson, E. D. & Verma, I. M. *Nature* **304**, 454–456 (1983).
- Curran, T. & Teich, N. M. *J. Virol.* **42**, 114–122 (1982).

Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity

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A chemically-synthesized gene and natural complementary DNA coding for human lymphotoxin were isolated and engineered for expression in Escherichia coli. Purified recombinant lymphotoxin shows cytotoxic activity on murine and human tumour cell lines in vitro and causes necrosis of certain murine sarcomas in vivo.

LYMPHOTOXIN was first characterized as a biological factor in mitogen-stimulated lymphocytes having anticellular activity on neoplastic cell lines¹⁻³. It has a spectrum of associated cytotoxic activities, including cytostasis of certain tumour cell lines and marked cytolysis of other transformed cells⁴⁻⁸. On primary cell cultures and normal cell lines, lymphotoxin shows little or no anticellular activity. This discrimination led to *in vivo* studies⁹⁻¹¹ suggesting that lymphotoxin has a potent antitumour activity.

Purification and subsequent characterization of lymphotoxin has proved difficult because of the small amount of active protein secreted by primary lymphocyte cultures. We reported recently the isolation and characteristics of lymphotoxin produced by the human lymphoblastoid cell line RPMI-1788 (ref. 12). Antibodies prepared against RPMI-1788-derived lymphotoxin neutralize all of the cytolytic activity produced by non-adherent lymphocytes¹³, suggesting that lymphotoxin from both sources is identical. Lymphotoxin is a glycoprotein with a relative molecular mass (M_r) 60,000–70,000 measured by molecular sieve chromatography^{12,13}, whereas monomeric lymphotoxin has a M_r of 25,000 (ref. 14); previous values of 20,000 (ref. 12) probably represent a degradation product. We report here the construction and expression in *Escherichia coli* of a DNA encoding human lymphotoxin and some of the *in vitro* and *in vivo* biological activities of this anticellular lymphokine.

Lymphotoxin gene design

Cultures of RPMI-1788 cells were induced with the tumour-promoting agent 4 β -phorbol-12 β -myristate 13 α -acetate (PMA) and produced 500–1,000 lymphotoxin units per ml, as determined in the murine fibroblast L-929 assay^{13,15,16}. Lymphotoxin was purified as described previously¹² and migrated on SDS-polyacrylamide gels as two bands of M_r 25,000 (95% of material) and 20,000 (5% of material). Microsequencing^{17,18} by the Edman degradation technique yielded sequence information on the intact molecule and on fragments produced by various enzymatic and chemical cleavages. A contiguous sequence of 155 residues was determined, but the carboxy-terminal region was difficult to determine because of the limited availability of material and hydrophobic nature of this region¹⁴. Three carboxy-terminal residues were determined by carboxypeptidase digestion (-Phe-Ala-Leu-COOH), but several residues in this region remained unknown¹⁴.

We constructed a synthetic gene containing an ATG translational initiation codon followed by codons for the 155 residues determined by microsequencing. This design assumed that the unknown carboxy-terminal residues would not be necessary for lymphotoxin activity. The gene design incorporated *E. coli* codon bias^{19,20} and substituted preferred human codons²¹ where no *E. coli* bias was apparent. The entire gene was assembled in three segments from 58 individual oligodeoxynucleotides

synthesized by the solid phase phosphite method^{22,23}. Extracts prepared from *E. coli* cultures containing the resulting expression plasmid pLTxBtrp1 were not active in the L-929 assay, suggesting that the carboxy-terminal residues not identified by protein sequencing are necessary for lymphotoxin activity.

Lymphotoxin cDNA isolation

The synthetic lymphotoxin gene was used as a probe to isolate the natural lymphotoxin complementary DNA sequence. RNA was isolated from a culture of non-adherent human peripheral blood lymphocytes stimulated with PMA, staphylococcal enterotoxin B and thymosin α_1 (refs 13, 24). This RNA was used to prepare a cDNA library in vector λ gt10 (ref. 25), which was screened²⁶ in conditions of low stringency²⁷ with a probe prepared from the N-terminal coding region (116 base pairs, bp, in length) of the synthetic lymphotoxin gene.

The DNA sequence of the hybridizing phage containing the longest cDNA insert (λ LT11) was determined by the dideoxy-chain termination method (ref. 28; Fig. 1). There is an open reading frame of exact amino acid homology with the determined lymphotoxin protein sequence¹⁴. The lymphotoxin cDNA structure is similar to that of other cDNAs which have been characterized, containing 79 bp of 5' untranslated and 626 bp of 3' untranslated sequence and the consensus polyadenylation addition sequence AATAAA²⁹ just upstream of the poly(A) tail. λ LT11 cDNA encodes 34 residues before the sequenced amino terminus which have the characteristics of a signal sequence³⁰.

Table 1 Antigenic similarity of natural and recombinant lymphotoxin as assessed by neutralization of cytolytic activity

Lymphotoxin pretreatment	% Cytolysis	
	Natural lymphotoxin	Recombinant lymphotoxin
Preimmune serum	100	100
Rabbit anti-lymphotoxin	0	0
Lymphotoxin monoclonal antibody lymphotoxin-B	0	0
55 °C, 1 h	100	100
80 °C, 1 h	0	0

Ten units of lymphotoxin were preincubated with 5 μ l (5 μ g) polyclonal or monoclonal antibody in a total volume of 0.1 ml for 1 h at 37 °C before assay on murine L-929 cells as described previously^{13,15}. The assay is sensitive to 0.4 U in 0.1 ml. Preimmune and hyperimmune rabbit antisera were obtained from a rabbit immunized with purified natural lymphotoxin derived from RPMI-1788 cells. Neutralizing monoclonal antibody was derived from a mouse immunized with natural lymphotoxin; 1 mg of lymphotoxin-B neutralizes ~4,000 U of natural or recombinant lymphotoxin.

Fig. 2 Characterization of the gene and RNA coding for human lymphotoxin. Lanes 1-4, Southern hybridization of human genomic DNA digested with restriction endonucleases. Human lymphocyte DNA (5 µg) prepared by the method of Blin and Stafford⁵⁴ was digested to completion with *EcoRI* (lane 1), *PvuII* (lane 2), *HindIII* (lane 3) or *PstI* (lane 4). The digested DNAs were electrophoresed on a 1% agarose gel and blotted to a nitrocellulose membrane³¹. A probe prepared⁵² from the coding region of the lymphotoxin cDNA was hybridized (10⁷ c.p.m.) with the filter for 16 h and then washed as described elsewhere^{24,26}. Lanes 5-7, the ethidium bromide staining pattern of RNAs electrophoresed through a denaturing agarose gel. The 10-µg RNA samples were electrophoresed through the gel (1.75% agarose, 0.025 M sodium citrate (pH 3.8), 6 M urea) for 7 h at 25 mA and 4 °C^{55,56}. The gel was stained in 5 µg ml⁻¹ ethidium bromide and photographed with an ultraviolet light source, then washed in 10 mM sodium phosphate (pH 7.0) to remove urea. RNA was transferred to nitrocellulose⁵⁷ and then hybridized and washed as above for the Southern blot (lanes 8-10). The samples are mRNA from RPMI-1788 cells (lanes 5, 8), induced adherent leukocyte (macrophage) cell RNA (lanes 6, 9) and induced non-adherent (lymphocyte) mRNA (lanes 7, 10). The mobility of the band observed in lanes 8 and 10 corresponds to an RNA 14S in size. 28S and 18S refer to ribosomal RNA size markers; numbers on the left indicate mobilities of *HindIII* A DNA fragments in kb.

We found 16 residues at the carboxy terminus encoded by the cDNA which had not been identified by protein sequencing. The three residues determined by carboxypeptidase digestion (Phe-Ala-Leu; ref. 14) are encoded by the cDNA immediately preceding the termination codon (Fig. 1). Because the 16 C-terminal codons were not present in the inactive synthetic lymphotoxin gene, a hybrid expression plasmid was prepared by splicing carboxy-terminal coding sequences from the cDNA into the synthetic gene (see Fig. 1 legend). Cultures containing the resulting expression plasmid, pLTtrp1, were found to contain cytolytic activity (5×10^3 units ml^{-1} when grown to $1.0 A_{550}$) when measured by the murine L-929 assay, confirming the cDNA sequence (Fig. 1) as that of human lymphotoxin.

We used ^{32}P -labelled lymphotoxin cDNA to analyse the structure of the lymphotoxin gene and RNA. Southern blots³¹ hybridized with the lymphotoxin cDNA probe suggest that human lymphotoxin is encoded by a single gene (Fig. 2). Northern hybridizations of RNAs from induced lymphocytes or RPMI-1788 cells demonstrate that a transcript of ~14S is produced from the lymphotoxin gene (Fig. 2) which agrees well with the length of the cDNA (1,329 bp).

Purification and characterization

Polyclonal antibodies prepared in rabbits against natural lymphotoxin¹³ neutralized all of the cytolytic activity contained in the recombinant cultures (Table 1). A murine monoclonal antibody (LT-B) derived to natural lymphotoxin (C.V.B. and T.S.B., unpublished results) is also capable of neutralizing completely the L-929 cytolytic activity of lymphotoxin from natural and recombinant sources (Table 1). The recombinant lymphotoxin

Fig. 1 Sequence of the lymphotoxin cDNA. Numbers above each line refer to amino acid position and numbers below each line to nucleotide position. The residue labelled 1 represents the first residue of lymphotoxin sequenced and is presumably the first amino-terminal residue of mature lymphotoxin. The first 34 residues (lower case lettering) represent a signal sequence. Residues 156–171 were not determined initially by protein sequencing. RNA was isolated⁴⁸ from the non-adherent⁴⁹ cell fraction of human peripheral blood lymphocytes 48 h after induction with phorbol myristate acetate (10 ng ml⁻¹), staphylococcal enterotoxin B (1 µg ml⁻¹), and thymosin-α₁ (1 µg ml⁻¹)^{13,24}. The RNA was passed over oligo(dT) and then used to prepare cDNA^{50,51}, which was cloned into λgt10 (ref. 25). Approximately 10,000 recombinant phage were plated on a 15-cm plate and screened by a low-stringency plaque hybridization method^{26,27}. A ³²P-labelled probe was prepared from the N-terminal coding segment (116 bp) by methods described previously⁵², using calf thymus DNA primers (PL Biochemicals). Duplicate nitrocellulose filters were hybridized as described elsewhere^{26,27} with 5 × 10⁷ c.p.m. of the probe in 20% formamide. The filters were washed twice in 0.3 M NaCl, 0.03 M sodium citrate, and 0.1% SDS at 37°C. Two phage hybridized with the probe and were plaque purified²⁶. The purified phage hybridized with probes prepared from the three individual segments of the synthetic gene. The cDNA inserts of the two hybridizing phage, λLT1 and λLT2, were subcloned into M13mp8 and sequenced by the dideoxy-chain termination method²⁸. A ³²P-labelled probe was prepared from the insert of λLT1 and used to screen 25,000 recombinant phage at high stringency²⁶. Twelve additional hybridizing phage were identified and the sequence of the longest insert, from λLT11, is presented in the figure. The longest open reading frame is translated starting at the first observed ATG.

Methods: A hybrid synthetic gene/natural cDNA expression plasmid was constructed as follows. A 685-bp fragment containing a *trp* promoter and DNA coding for 125 residues of the synthetic gene were isolated by digesting plasmid pLTXBtrp1 with *EcoRI* and *PstI*. A 301-bp fragment containing DNA coding for the C-terminal 51 amino acids of lymphotoxin was isolated by digesting the subcloned cDNA of λ LT1 with *EcoRI* and *PstI* (these sites are shown above at nucleotide positions 554 and 855). These fragments were isolated by electrophoresis on 5% polyacrylamide gels and electroelution. The fragments were ligated into *EcoRI*-cleaved pBR322 treated with bacterial alkaline phosphatase⁵³ to reduce background transformants. The resulting expression plasmid, pLTtrp1, was characterized by restriction endonuclease digestion and DNA sequencing.

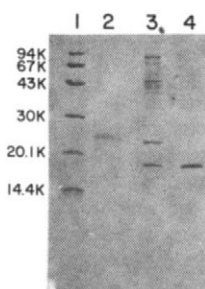


Fig. 3 Reducing (2-mercaptoethanol) 15% SDS-polyacrylamide gel of proteins stained with Coomassie blue. Lane 1, size markers (sizes ($M_r \times 10^3$) indicated to the left); lane 2, natural lymphotoxin purified as previously described¹²; lane 3, proteins from extracts of pLTtrp1-containing *E. coli* selectively precipitated with 40% saturated ammonium sulphate; lane 4, eluate from a Sepharose/monoclonal antibody lymphotoxin-B column. Conditions of immunoaffinity purification are briefly as follows: The monoclonal antibody purified from ascites fluid by ion exchange chromatography was coupled to cyanogen bromide-activated Sepharose at a concentration of 2 mg ml⁻¹ resin. A 20-ml column was equilibrated consecutively with TBS (0.05 M Tris-HCl, pH 7.0, 0.15 M sodium chloride, 2 mM EDTA); 0.1 M acetic acid, pH 4.5, 150 mM NaCl (elution buffer), then TBS. A 40% saturated ammonium sulphate precipitate of *E. coli* pLTtrp1 lysate was dissolved in 0.1 M Tris-HCl, pH 7.4 and 5 mM EDTA and loaded onto the column at a rate of one column volume per h. Following extensive washing with TBS containing 0.05% Tween-20, specifically-bound material was eluted and immediately adjusted to pH 7.8 with 0.1 volume 1 M Tris-HCl, pH 8.5 and stored at 4°C. The specific bioactivity of this purified lymphotoxin was 2.5×10^7 U mg⁻¹, where 1 U of lymphotoxin is defined as the amount required to achieve 50% lysis of 12,000 murine L-929 cells in a standard microtitre well.

activity also exhibited thermostability similar to natural lymphotoxin (ref. 16 and Table 1).

The recombinant lymphotoxin was purified on a monoclonal antibody (LT-B) column (Fig. 3). The mobility of the eluted lymphotoxin corresponds to M_r 18,000, consistent with the predicted value of 18,660 based on the deduced amino acid sequence. Recombinant lymphotoxin produced in *E. coli* clearly causes cytolysis of murine L-929 cells, similar to lymphotoxin derived from RPMI-1788 cells. Anticellular activity was measured *in vitro* by plating tumour cells at a low density and then monitoring their growth in the presence of lymphotoxin. We found that the recombinant and natural lymphotoxin had

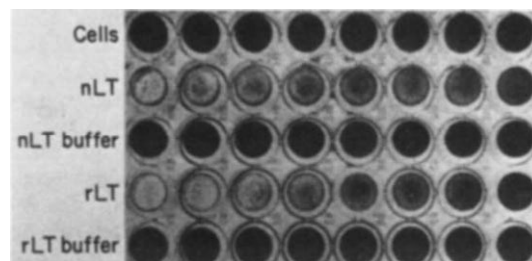


Fig. 4 Cytotoxic activity of recombinant and natural lymphotoxin on ME180 cells. Natural lymphotoxin (second row, nLT) and recombinant lymphotoxin (fourth row, rLT) were added to 96-well flat-bottom microtitre plates at 1×10^4 U per well and serially diluted twofold, across the plate from left to right. The final concentration in the far right well is 78 U. Buffer 1 and buffer 2 controls (as in Table 2) were added to the third and fifth rows, respectively. ME180 cells were subsequently added at a concentration of 5×10^4 per well and the plates incubated at 37°C. After 72 h the supernatants were removed and the cells stained with crystal violet. Results show significant cytotoxic activity of lymphotoxin on ME180 cells (second and fourth rows) when compared with buffer controls (third and fifth rows) and ME180 cells alone (top row).

a significant antiproliferative effect on the human tumour cell line ME-180, a human cervical carcinoma (Fig. 4). A normal lung fibroblast line, WI-38, was not sensitive to lymphotoxin (ref. 16).

Recombinant and natural lymphotoxin were tested in an *in vivo* tumour necrosis assay³² which involves growing methylcholanthrene-induced (MethA) sarcomas in susceptible mice and then injecting the tumours directly with lymphotoxin or control samples. After 20–24 h, the mice are killed and the tumours removed and histologically scored for the extent of necrosis³². Both recombinant and natural lymphotoxin caused significant necrosis of MethA sarcomas *in vivo* (Table 2).

Discussion

We report here the cloning and expression of DNA sequences encoding human lymphotoxin. The various M_r s of lymphotoxin described previously^{5,33,34} probably do not represent a family of closely-related proteins, because hybridization studies suggest that human lymphotoxin is encoded by a single gene (Fig. 2) and polyclonal or monoclonal antibodies neutralize all of the L-929 anticellular activity produced from RPMI-1788 cells and induced non-adherent lymphocyte cultures¹³. These various size species could result from differential processing of the lymphotoxin mRNA or protein, but a more probable explanation is that this heterogeneity results from aggregation and/or degradation phenomena. As determined by protein sequencing and cDNA cloning, mature lymphotoxin is 171 residues in length (M_r 18,660); N-linked glycosylation^{14,35} at the observed potential Asn-X-Ser site (residues 62–64 of Fig. 2a) probably accounts for the increased size (M_r 25,000) observed for natural lymphotoxin. The absence of carbohydrate on recombinant lymphotoxin does not seem to affect its cytotoxic activity, as the specific activity on L-929 cells of lymphotoxin produced by recombinant means (2.5 – 13×10^7 U mg⁻¹) is approximately that reported for natural lymphotoxin (4×10^7 U mg⁻¹)¹⁴. The 25,000 M_r form of lymphotoxin contains 23 extra N-terminal residues when compared with the 20,000 M_r lymphotoxin¹⁴. Production of lymphotoxin by recombinant methods should eliminate the heterogeneous nature of the lymphotoxin amino terminus and allow the isolation of lymphotoxin with a distinct amino terminal end.

The amino acid sequence of lymphotoxin was compared with the sequences of the lymphokines γ -interferon²⁴, interleukin-2 (ref. 36), and interleukin-3 (ref. 37). No significant homologies were observed even though all these lymphokines are secreted from induced lymphocytes and have similar sizes to lymphotoxin. There is at least one cytolytic factor of haematopoietic origin distinct from lymphotoxin, the cytokine tumour necrosis

Table 2 Necrosis of MethA sarcoma *in vivo* by recombinant and natural lymphotoxin

Treatment	Sarcoma necrosis score			
	+++	++	+	-
Natural lymphotoxin, 25,000 U	4	-	-	-
Natural lymphotoxin, 10,000 U	4	-	-	-
Buffer 1 control	-	-	-	10
Recombinant lymphotoxin 200,000 U	14	2	2	-
Recombinant lymphotoxin, 25,000 U	3	-	-	1
Recombinant lymphotoxin, 10,000 U	3	-	1	-
Buffer 2 control	-	-	1	9

Tumour necrosis activity was assayed after intratumoral injection of lymphotoxin (or buffer control) into 7–10 day MethA transplants in CB6F1 mice. At 24 h after the injection, mice were killed and tumours scored histologically for haemorrhagic necrosis. Natural lymphotoxin was purified as previously described¹² and recombinant lymphotoxin was immunoaffinity purified (Fig. 3). Natural lymphotoxin was in buffer 1 (0.01 M Tris-HCl, 0.05 M (NH₄)₂ HCO₃, pH 8.0) and recombinant lymphotoxin was in buffer 2 (0.15 M NaCl, 0.1 M sodium acetate, 0.1 M Tris-HCl, pH 7.8). +++, Maximum response, 50–75% of the tumour mass markedly necrotic after 24 h; ++, moderate response, 25–50% haemorrhagic necrosis; +, minimal response of <25% haemorrhagic necrosis; -, no visible necrosis.

factor (TNF)^{32,38-41}. This factor causes necrosis of MethA(a) sarcoma *in vivo*; it is also active in causing lysis of murine L-929 cells *in vitro*^{32,39}. TNF activity on L-929 cells is not inhibited by neutralizing antibodies specific for lymphotoxin¹³, however, and appears to have distinct biochemical properties^{39,40}. Furthermore, the lymphotoxin cDNA probe failed to hybridize on Northern blots to mRNA from induced macrophages producing cytolytic activity (Fig. 2). The isolation of a TNF cDNA clone further demonstrates that lymphotoxin and TNF are distinct molecules (see accompanying article⁴²).

A cytolytic factor derived from B-cell lines which displays *in vitro* and *in vivo* anticellular activity has been described⁴³; this has been designated 'tumour necrosis factor' based on its activity in the MethA sarcoma assay. This activity probably results from lymphotoxin, however, because it has similar biological activities, biochemical properties and is made by the cell line (RPMI-1788) used in this study for the purification of lymphotoxin. Natural killer cells also can be induced to secrete an anticellular factor^{44,45}. The lymphotoxin gene probe and lymphotoxin-specific antibodies will be useful in determining the

relationship of this natural killer cell cytotoxic factor to lymphotoxin.

Lymphotoxin has been reported to act synergistically with α -interferon⁴⁶ and γ -interferon^{13,16,47} *in vitro* and *in vivo*. The potent antitumour activity of γ -interferon or lymphotoxin in natural preparations may be a result of the synergistic activity when both lymphokines are present¹³. The ready availability of lymphotoxin produced via recombinant methods will aid the biological characterization of this anticellular lymphokine. It will also help to define the antitumour mechanism of lymphotoxin, as well as its role *in vivo* in the regulation of the immune system and its interaction with other lymphokines.

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- Ruddle, N. H. & Waksman, B. H. *J. exp. Med.* **128**, 1267-1279 (1968).
- Granger, G. A. & Kolb, W. P. *J. Immunol.* **101**, 111-120 (1968).
- Rosenau, W. *Fedn Proc.* **27**, 34-38 (1968).
- Sawada, J., Shiorri-Nakano, K. & Osawa, T. *Jap. J. exp. Med.* **46**, 263-267 (1976).
- Granger, G. A., Yamamoto, R. S., Fair, D. S. & Hiserodt, J. C. *Cell. Immun.* **38**, 388-402 (1978).
- Rundell, J. O. & Evans, C. H. *Immunopharmacology* **3**, 9-18 (1981).
- Granger, G. A., Yamamoto, R. S., Devlin, J. J. & Klostergaard, J. *Lymphokine Res.* **1**, 45-49 (1982).
- Ruddle, N. H., Powell, M. B. & Conta, B. S. *Lymphokine Res.* **2**, 23-21 (1983).
- Khan, A. *et al. Proc. Soc. exp. Biol. Med.* **169**, 291-294 (1982).
- Papernaster, B. W. *et al. Cancer* **45**, 1248-1253 (1980).
- Ransom, J. H., Evans, C. H. & DiPaolo, J. A. *J. natn. Cancer Inst.* **69**, 741-744 (1982).
- Aggarwal, B. B., Moffat, B. & Harkins, R. N. *J. biol. Chem.* **259**, 686-691 (1984).
- Stone-Wolf, D. S. *et al. J. exp. Med.* **159**, 828-843 (1984).
- Aggarwal, B. B., Henzel, W. J., Moffat, B., Kohr, W. J. & Harkins, R. N. *J. biol. Chem.* (in the press).
- Spofford, B. T., Daynes, R. A. & Granger, G. A. *J. Immunol.* **112**, 2111-2116 (1974).
- Aggarwal, B. B., Moffat, B., Lee, S. H. & Harkins, R. N. in *Thymic Hormones and Lymphokines* (ed. Goldstein, A.) 235-245 (Plenum, New York, 1984).
- Kohr, W. J., Keck, R. & Harkins, R. N. *Analyt. Biochem.* **122**, 348-359 (1982).
- Rodriguez, H., Kohr, W. J. & Harkins, R. N. *Analyt. Biochem.* (in the press).
- Ikemura, T. *J. molec. Biol.* **151**, 389-409 (1981).
- Grosjean, H. & Fiers, W. *Gene* **18**, 199-209 (1982).
- Wain-Hobson, S., Nussinov, R., Brown, R. J. & Sussman, J. L. *Gene* **13**, 355-364 (1981).
- Beaucage, S. L. & Caruthers, M. H. *Tetrahedron Lett.* **22**, 1859-1862 (1981).
- Matteucci, M. & Caruthers, M. H. *J. Am. chem. Soc.* **103**, 3185-3190 (1981).
- Gray, P. W. *et al. Nature* **295**, 503-508 (1982).
- Huynh, T., Young, R. & Davis, R. in *Practical Approaches in Biochemistry* (ed. Grover, D.) (IRL, Oxford, in the press).

- Maniatis, T. *et al. Cell* **15**, 687-701 (1978).
- Gray, P. W. & Goeddel, D. V. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5842-5846 (1983).
- Smith, A. J. H. *Meth. Enzym.* **65**, 560-580 (1980).
- Proudfoot, N. W. & Brownlee, G. G. *Nature* **263**, 211-214 (1976).
- Kreil, G. A. *Rev. Biochem.* **50**, 317-348 (1981).
- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Carswell, E. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 3666-3670 (1975).
- Walker, S. M., Lee, S. C. & Lucas, Z. J. *J. Immunol.* **116**, 807-815 (1976).
- Ross, M. W. *et al. J. Immunol.* **123**, 325-331 (1978).
- Winzler, R. J. in *Hormonal Proteins and Peptides* (ed. Li, C. H.) 1-15 (Academic, New York, 1973).
- Taniguchi, T. *et al. Nature* **302**, 305-310 (1983).
- Fung, M. C. *et al. Nature* **307**, 233-237 (1984).
- Ostrove, J. M. & Gifford, G. E. *Proc. Soc. exp. Biol. Med.* **160**, 354-358 (1979).
- Mannel, D. N., Moore, R. N. & Mergenhausen, S. E. *Infect. Immun.* **30**, 523-530 (1980).
- Green, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 381-385 (1976).
- Kull, F. C. & Cuatrecasas, P. *J. Immunol.* **126**, 1279-1283 (1981).
- Pennica, D. *et al. Nature* **312**, 724-729 (1984).
- Williamson, B. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 5397-5401 (1983).
- Wright, S. C. & Bonavida, B. *J. Immunol.* **126**, 1516-1521 (1981).
- Farrar, E. & Targan, S. R. *J. Immunol.* **130**, 1252-1256 (1983).
- Williams, T. W. & Bellanti, J. A. *J. Immunol.* **130**, 518-520 (1983).
- Lee, S. H., Aggarwal, B. B., Rinderknecht, E., Assisi, F. & Chin, H. *J. Immunol.* (in the press).
- Berger, S. B. & Birkenmeier, C. S. *Biochemistry* **18**, 5143-5149 (1979).
- Kumagi, K. *et al. J. immun. Meth.* **29**, 17-25 (1979).
- Goeddel, D. V. *et al. Nature* **287**, 411-416 (1980).
- Wickens, M. P., Buell, G. N. & Schimke, R. T. *J. biol. Chem.* **253**, 2483-2495 (1978).
- Taylor, J. M., Illmensee, R. & Summers, S. *Biochim. biophys. Acta* **442**, 324-330 (1976).
- Ullrich, A. *et al. Science* **196**, 1313-1319 (1977).
- Blin, N. & Stafford, D. W. *Nucleic Acids Res.* **3**, 2303-2308 (1976).
- Lehrach, H., Diamond, D., Wozney, J. M. & Boedtker, H. *Biochemistry* **16**, 4743-4751 (1977).
- Lynch, K. R., Pennica, D., Ennis, H. L. & Cohen, P. S. *Virology* **98**, 251-254 (1979).
- Thomas, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205 (1980).

Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin

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Human tumour necrosis factor has about 30% homology in its amino acid sequence with lymphotoxin, a lymphokine that has similar biological properties. Recombinant tumour necrosis factor can be obtained by expression of its complementary DNA in Escherichia coli and induces the haemorrhagic necrosis of transplanted methylcholanthrene-induced sarcomas in syngeneic mice.

TUMOUR necrosis factor (TNF) has been associated with *in vitro* and *in vivo* killing of tumour cells. This activity was discovered originally in the sera of mice and rabbits injected first with *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG) or other immunostimulatory agents, and subsequently

with endotoxin^{1,2}. Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplanted tumours in mice^{1,2}. TNF-like activity has also been detected in the media of BCG/endotoxin-induced monocyte cultures (reviewed in ref. 2) and mitogen-stimulated peripheral

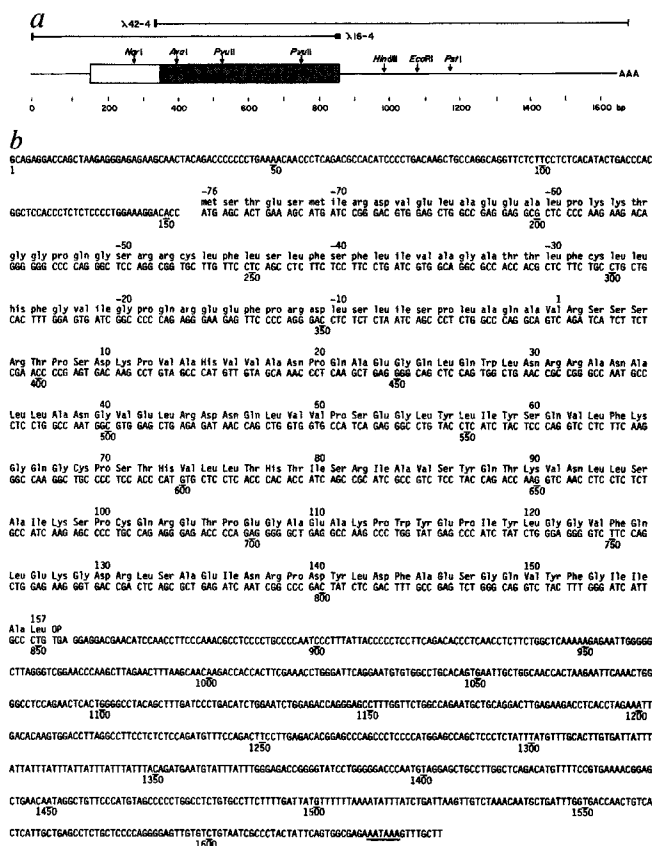


Fig. 1 TNF cDNA sequences and predicted amino acid sequence. **a**, Schematic representation of human TNF cDNA clones. Overlapping clones λ 42-4 and λ 16-4 used in sequence determination and a schematic diagram of the complete cDNA structure are shown. Line, untranslated sequences; boxes, coding sequences; white portion, sequences encoding the signal peptide; shaded regions code for mature TNF. The black box on the 3' end of clone λ 16-4 indicates that this clone was obtained by specific priming. **b**, Nucleotide sequence and deduced amino acid sequence of human TNF cDNA. Numbers above each line refer to amino acid positions and numbers below each line refer to nucleotide positions. The amino acid labelled 1 represents the first amino acid of mature TNF¹⁶. The 76 amino acids preceding this position are indicated by lower case lettering. Sequence underlined indicates the polyadenylation recognition site²⁷.

Methods: **a**, Total RNA was extracted²⁰ from HL-60 cultures 4 h after PMA induction and poly(A)-containing RNA was purified on oligo(dT)-cellulose⁴³. Double-stranded cDNA was prepared by oligo(dT) priming¹⁹ using 7.5 μ g mRNA and fractionated on a 6% polyacrylamide gel. 700 ng cDNA >600 bp was recovered by electroelution. Synthetic *Eco*RI adaptors⁴⁴ were ligated to 20 ng cDNA before ligating into λ gt10 (ref. 26). 200,000 cDNA clones were obtained. The same conditions were used to prepare a specifically-primed cDNA library of 200,000 clones using as primer the hexadecanucleotide dTGGATGTTCTGCTCTCC (complementary to nucleotides 855-870). Plaque screening⁴⁵, ³²P-radiolabelling of synthetic 42-mer probe²⁵ and hybridizations²² were performed. **b**, DNA sequencing was performed by the dideoxynucleotide chain termination procedure⁴⁶⁻⁴⁸. The cDNA insert of λ 42-4 consists of nucleotides 337-1643 and the cDNA insert of λ 16-4 consists of nucleotides 1-870.

blood leukocytes (PBLs)³.

TNF activity is cytolytic or cytostatic against many transformed cell lines *in vitro* without obvious species specificity, yet has no known effect on normal mouse embryo fibroblasts or non-transformed cell lines^{1,2,4-8}. Activated macrophages may constitute the major cellular origin of TNF^{1,5,9,10}, providing an important criterion for distinguishing this factor from the lymphoid cell-derived cytotoxin, lymphotoxin¹¹. The primary structure of lymphotoxin was determined recently by protein sequencing¹² and complementary DNA cloning (see accompanying article¹³).

Table 1 Human TNF production by various cell populations and cell lines

Cell source	Inducing agent(s)	Cytotoxic activity (U ml ⁻¹)	
		TNF	Lymphotoxin
Unfractionated PBLs	None	<8	<8
	LPS	20	<8
	BCG	82	<8
	BCG/LPS	86	<8
	BCG/LPS/PMA	140	<8
	PMA	280	<8
PBLs (adherent cells)	SEB/T α_1	100	10
	SEB/T α_1 /PMA	1,600	200
	None	<8	<8
	BCG/LPS	350	<8
	SEB/T α_1 /PMA	590	<8
	None	<8	<8
PBLs (non-adherent cells)	BCG/LPS	<8	<8
	SEB/T α_1 /PMA	<8	350
	None	<8	<8
HL-60	None	<8	<8
	PMA	380	<8
U-937	None	<8	<8
	PMA	32	<8

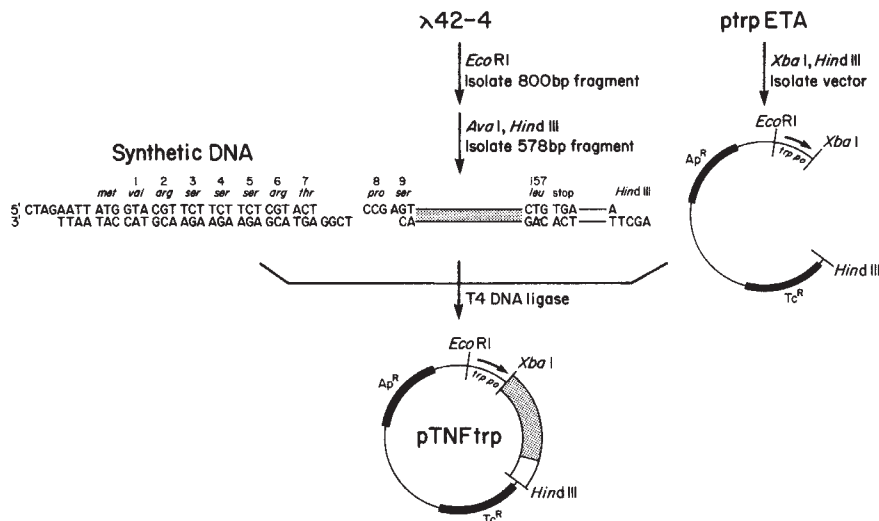
PBLs were obtained from plateletpheresis residues (Boston Red Cross) by Ficoll-Hypaque centrifugation³. Separation of PBLs into adherent and non-adherent populations was performed as described previously⁴¹. HL-60 (CCL 240) and U-937 cell lines (CRL 1593) were obtained from the American Type Culture Collection. Cells were suspended at 5×10^6 cells ml⁻¹ in RPMI 1640 media containing 10% fetal bovine serum. Cultures were induced with one or more of the following agents: 2×10^5 organisms per ml of BCG (Calbiochem-Behring), 20 μ g ml⁻¹ *Salmonella typhimurium* lipopolysaccharide (LPS, Sigma), 1 μ g ml⁻¹ staphylococcal enterotoxin B (SEB, Sigma), 1 μ g ml⁻¹ thymosin α_1 (T α_1)⁴² and 10 ng ml⁻¹ PMA (P-L Biochemicals). Cell-free supernatants were collected 24 h after induction except for the BCG/LPS and BCG/LPS/PMA treatments; for these two inductions a 24-h BCG stimulation was followed by an additional 24-h treatment with LPS and LPS/PMA, respectively. Samples were assayed for cytotoxic activity on mouse L-929 fibroblasts as described previously¹¹. The activities shown represent TNF-specific or lymphotoxin-specific units as determined after antibody neutralization at 4 °C for 4 h before assay. The units indicated were obtained from one representative donor in the case of the PBLs and from a single experiment when cell lines were used. Rabbit anti-human TNF antiserum was prepared against partially purified TNF from PBLs (L. Svedersky and T. Bringman, unpublished results). Rabbit anti-human lymphotoxin antiserum was prepared against purified human lymphotoxin from RPMI 1788 lymphoblastoid cells¹¹.

Here we identify a cell line with monocyte-like characteristics providing a source for human TNF and its messenger RNA. cDNA clones were isolated that encode a polypeptide related structurally to lymphotoxin. This cDNA was engineered to direct the synthesis of a relative molecular mass (M_r) 17,000 protein in *E. coli* with the immunological characteristics as well as *in vitro* and *in vivo* biological properties of natural human TNF.

A human TNF-producing cell line

We isolated PBLs by Ficoll-Hypaque density centrifugation and fractionated them into adherent monocytic and non-adherent lymphocytic fractions. After stimulation with BCG and endotoxin (lipopolysaccharide, LPS), we detected an activity cytotoxic to murine L-929 cells in the culture media of unfractionated mononuclear cells and monocytes (Table 1). No cytotoxic activity was produced by the non-adherent cells following the same BCG/LPS induction procedure. The failure of rabbit anti-human lymphotoxin antibodies to neutralize the cytotoxic activity demonstrates its difference from lymphotoxin. Moreover, the results of previous *in vivo* studies using BCG/LPS induction procedures^{1,2} demonstrate that the activity can probably be attributed to TNF. Antiserum raised against partially purified PBL-produced TNF completely neutralized this activity (Table 1).

Fig. 2 Construction of a plasmid coding for the direct expression of mature human TNF in *E. coli*. The recombinant phage λ 42-4 (10 μ g) was digested with *Eco*RI and the 800-bp fragment containing the entire TNF coding region was isolated. TNF digestion with *Ava*I and *Hind*III gave a 578-bp fragment coding for amino acids 8–157. Two synthetic complementary deoxyoligonucleotides¹⁷, 5'-dCTAGAAT-TATGGTACGTTCTTCTCTCTCGTACT and 5'-dTCCGGAGTACGAGAAGAAGAACGTACCATAAT, were designed to code for amino acids 1–7 of TNF, preceded by an ATG translational initiation codon, and to contain an *Xba*I cohesive terminus. The choice of codons for the first six amino acids of TNF was based on *E. coli* codon usage preferences⁴⁹. An AATT sequence was incorporated upstream of the ATG to maximize expression by giving optimal spacing between the initiation codon and the *trp* leader Shine-Dalgarno sequence⁵⁰. The pBR322-derived plasmid pTrpETA⁵¹ was cleaved with *Hind*III and *Xba*I and the large fragment recovered by electroelution. The *Ava*I–*Hind*III fragment and the two synthetic deoxyoligonucleotides were inserted into the plasmid pTNFtrp expression vector to give the plasmid pTNFtrp. The methods used to assemble the fragments and verify the construction of pTNFtrp have been described previously^{19,20}. *E. coli* W3110/pTNFtrp was grown in M9 medium containing 5 μ g ml⁻¹ tetracycline to 0.2 A_{550} units. Indole acrylic acid was added to a concentration of 20 μ g ml⁻¹. The cells were collected at A_{550} = 1.0 and washed with cold PBS. The final cell pellet was resuspended in 1 ml PBS, sonicated on ice for 30 s and the resulting extract diluted in PBS for assay on L-929 cells¹¹.



Yields of adherent cells from peripheral blood were low and the levels of TNF produced were variable and donor-dependent; we therefore tested alternative induction schemes for the production of TNF from total PBLs (Table 1). An increase in cytotoxic activity was observed when the PBLs were co-stimulated with *Staphylococcus* enterotoxin B, desacetyl-thymosin- α_1 and the tumour-promoting agent 4 β -phorbol 12 β -myristate 13 α -acetate (PMA). However, antibody neutralization experiments demonstrated that a significant portion of measured activity was lymphotoxin. Therefore, we screened a number of transformed cell lines of haematopoietic origin for their ability to synthesize TNF. Activity which could be neutralized by anti-TNF antiserum was detected following PMA treatment in two monocytic-like cell lines, HL-60, derived from a promyelocytic leukaemia¹⁴, and U-937, derived from a histiocytic lymphoma¹⁵ (Table 1). The HL-60 cell line consistently produced higher TNF titres (300–400 U ml⁻¹ 24 h after induction) than the U-937 cell line (<100 U ml⁻¹). A time course of TNF synthesis by HL-60 cultures indicated that measurable activity was detected 2 h after PMA treatment (data not shown). Therefore the HL-60 cell line was selected for future experiments; supernatants were collected 16–24 h after induction for protein purification and 4-h inductions were used when cells were collected for RNA isolation.

TNF cDNA clone identification

Human TNF was purified to homogeneity from filtrates of PMA-stimulated HL-60 cell cultures (see ref. 16). A single component of M_r 17,000 was observed when the purified TNF was analysed by SDS-gel electrophoresis in reducing conditions. To obtain amino acid sequence information, tryptic peptides of TNF were prepared and separated by reverse-phase HPLC.

The preliminary sequence Glu-Thr-Pro-Glu-Gly-Ala-Glu-Ala-Lys-Pro-Trp-Tyr-Glu-Lys was determined for the first tryptic fragment (TD-6) analysed. A single synthetic 42-base long deoxyoligonucleotide (42-mer) which could code for this amino acid sequence was chemically synthesized¹⁷ for use as a hybridization probe. The design of the probe sequence (dGAAACCCCTGAAGGGGCTGAAGCCAAGCCCTGGTATGAAAAG) was based on published human codon usage frequencies¹⁸ and the codon bias of human γ -interferon¹⁹, tissue-type plasminogen activator²⁰ and lymphotoxin¹³. The general usefulness of this 'long probe' approach has been demonstrated recently by the identification of several cloned genomic DNA sequences^{21–23} and cDNAs^{24,25}.

An oligo(dT)-primed HL-60 cDNA library of ~200,000 clones prepared in λ gt10 (ref. 26) was screened with the ³²P-labelled 42-mer. The nine distinct phage which gave positive signals with this probe were hybridized with 'induced' and 'non-induced' ³²P-labelled cDNA probes¹⁹ prepared using poly(A) mRNA obtained from 4-h PMA-treated and untreated HL-60 cultures, respectively. Seven of these recombinant phage DNAs hybridized weakly to the induced probe but did not hybridize to the uninduced probe, as expected for authentic TNF cDNAs. Restriction endonuclease mapping indicated that these seven cDNA clones were related to each other and that the phage λ 42-4 contained the longest cDNA insert.

TNF cDNA sequence

We determined the sequence of the 1,300 base pair (bp) cDNA insert of phage λ 42-4 (nucleotides 337–1,643; Fig. 1). Alignment of the cDNA sequence with the 42-mer probe sequence gave the proper reading frame of the cDNA and demonstrated that it did indeed encode TNF. Of the 14 amino acids (residues 104–117, Fig. 1) assigned to tryptic peptide TD-6 on the basis of preliminary protein sequence, 13 were correct; the only discrepancy was in the last amino acid (position 117) where the cDNA sequence encodes a proline residue rather than the predicted lysine. Despite this difference, the hybridization of the synthetic probe to the TNF cDNA clone was successful, as the 42-mer matched the cDNA sequence in 34 of the first 38 positions, including a stretch of 17 consecutive homologous nucleotides (nucleotides 711–727; Fig. 1).

The assignment of valine (residue 1, Fig. 1) as the first residue of mature TNF was based on NH₂-terminal protein sequence analysis of the intact molecule (Val-Arg-Ser-Ser-Ser-...) ¹⁶. There are 156 amino acids encoded after this valine before an in-phase termination codon occurs. The coding region of TNF is followed by 792 nucleotides of 3' untranslated sequence containing the hexanucleotide AATAAA (position 1,630–1,635) which precedes the site of polyadenylation in most eukaryotic mRNAs²⁷.

Additional confirmation that this sequence codes for TNF was obtained by determining the amino acid sequence of nine tryptic peptides of natural HL-60 TNF and several peptides generated by digestion with *S. aureus* V8 protease and chymotrypsin¹⁶. The M_r of 17,356 calculated for the mature TNF monomer from the cDNA sequence agrees closely with the value obtained for natural TNF by SDS-polyacrylamide gel electrophoresis and amino acid composition¹⁶. These results and

Table 2 Necrosis of MethA sarcoma *in vivo* by TNF

Treatment	Necrotic response			
	+++	++	+	-
	No. of mice			
PBS, i.l.	0	1	1	9
PBS, i.p.	0	0	1	4
PBS, i.m.	0	0	1	4
<i>E. coli</i> LPS, i.l.	0	0	1	9
HL-60 TNF, i.l.	5	1	1	0
rTNF, i.l.	7	5	0	0
rTNF, i.p.	2	1	2	0
rTNF, i.m.	2	2	1	0

(BALB/c×C57BL/6)_F₁ female mice were injected intradermally with 5×10^5 BALB/c MethA sarcoma cells. Ten days later, the tumours (0.75 cm average diameter) were injected intralesionally (i.l., 1×10^5 U), intraperitoneally (i.p., 5×10^5 U) or intramuscularly (i.m., 5×10^5 U) with TNF in a total volume of 0.1 ml PBS. At 24 h after TNF treatment the tumours were excised, sectioned and scored for haemorrhagic necrosis by visual and histological examination as described previously¹. In the maximum response (+++) 50–75% of the tumour mass is markedly necrotic after 24 h; ++ denotes a moderate response, that is 25–50% haemorrhagic necrosis; +, a minimal response of <25% haemorrhagic necrosis; –, tumours showed no visible necrosis. Natural TNF was purified from HL-60 cultures as described elsewhere¹⁶. Recombinant TNF (rTNF) was purified from *E. coli* W3110/pTNFtrp to a purity of >95% and a specific activity of $\sim 10^8$ units mg^{-1} (T. Bringman, unpublished results).

the absence of any potential *N*-glycosylation sites in the deduced amino acid sequence suggest that TNF is not a glycoprotein. These data suggest also that TNF may occur naturally in multimeric form, as the M_r estimated previously for human TNF ranged from 34,000–140,000 (refs 6, 28). There are two cysteine residues (positions 69 and 101) in TNF which are probably involved in a single intramolecular disulphide bond¹⁶.

The cDNA clone λ 42-4 contains the entire coding region of mature TNF but lacks a complete signal peptide coding sequence and initiation codon. To obtain the missing sequence information, a specifically-primed cDNA library was prepared (see Fig. 1 legend) and screened with the ³²P-labelled λ 42-4 cDNA insert. A cDNA clone (λ 16-4) was identified which contained an insert extending 337 bp further 5' than the λ 42-4 insert (Fig. 1).

From an analysis of the TNF cDNA sequence, it seems that TNF is synthesized initially as part of a larger precursor (pre-TNF). Starting at the 5' end of the cDNA, 125 nucleotides of non-translated sequence are followed by a methionine codon and an open reading frame of 233 amino acids. This AUG is preceded by termination codons in all three frames, suggesting that it is the initiation codon. Furthermore, the sequence context of this AUG conforms closely to the CC₆CCAUGG proposed²⁹ as a consensus sequence for eukaryotic initiator sites.

The presequence of 76 residues is most probably involved in the secretion of TNF as it is not observed on the mature TNF polypeptide and contains an unusually long hydrophobic region of 26 amino acids (residues –46 to –21). Typically, signal peptides involved in protein secretion are only 20–30 amino acids long^{30,31}. However, a signal sequence for the Rous sarcoma virus envelope glycoprotein³² is atypically long (63 residues) and contains also many charged amino acids at its amino terminus, such as pre-TNF. It is interesting to note the presence of Arg-Arg and Lys-Lys dipeptides in the first 30 amino acids of the TNF pre-sequence, as pairs of basic amino acids often serve as cleavage sites for the release of physiologically-important peptides from precursor molecules^{33–36}.

We used the ³²P-labelled λ 42-4 cDNA insert to examine TNF gene structure and mRNA size. Results from Southern³⁷ hybridizations indicate that only a single gene for TNF is present in the human genome. Northern hybridization analysis³⁸ shows that a single mRNA species ~ 1.8 S in size is synthesized in PMA-induced HL-60 cultures and BCG/LPS-treated macrophages isolated from human PBLs. This provides additional evidence that the same cytotoxin is produced from both

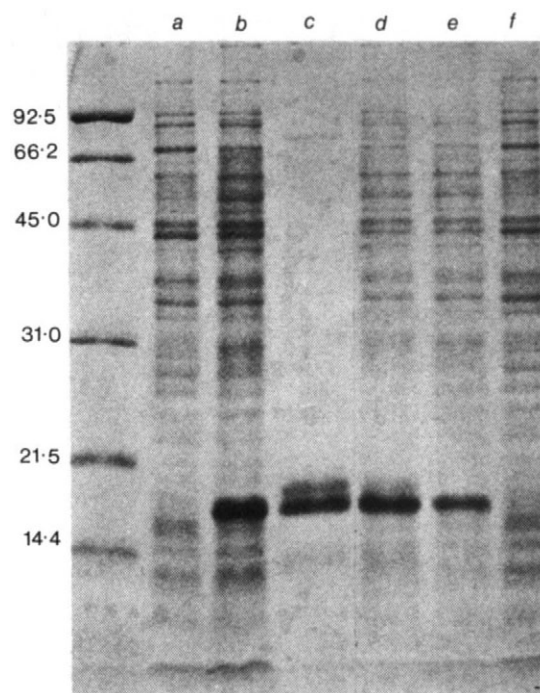


Fig. 3 SDS-polyacrylamide gel electrophoresis of human TNF synthesized in *E. coli*. *E. coli* K-12 strain W3110, transformed with pTNFtrp or pBR322, was grown in M9 medium containing $5 \mu\text{g ml}^{-1}$ tetracycline. Cells were collected, lysed in 2% SDS, 1% β -mercaptoethanol and precipitated with 10 volumes of cold acetone. Samples were electrophoresed on a 12.5% SDS-polyacrylamide slab gel using the buffer system of Maizels⁵² and the gel stained with Coomassie brilliant blue. The left lane contains protein M_r standards ($\times 10^{-3}$): phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,500). Lanes *a*, *f*, cell lysates of *E. coli* W3110/pBR322; lanes *b*, *e*, cell lysates of *E. coli* W3110/pTNFtrp; lane *c*, partially purified human TNF isolated from the HL60 cell line¹⁶; lane *d*, mixture of the *E. coli* W3110/pTNFtrp cell lysate and the HL60-derived, purified TNF.

cell sources and suggests that the TNF cDNA sequence shown in Fig. 1 represents a nearly full-length copy of TNF mRNA. No hybridization was detected to mRNA isolated from uninduced cultures (data not shown).

TNF synthesis in *E. coli*

Proof that the cDNA described here encodes TNF requires the demonstration that it can direct the synthesis of a gene product with the properties of authentic human TNF. To allow characterization of the protein encoded by the cloned cDNA, we engineered the TNF cDNA sequence for direct expression in *E. coli* (Fig. 2). In the resulting expression plasmid, pTNFtrp, the TNF DNA sequence is under the transcriptional control of a 300-bp DNA fragment of the *E. coli* *trp* operon containing the *trp* promoter, operator and Shine-Dalgarno sequence of the *trp* leader peptide.

Total extracts of *E. coli* K-12 strain W3110 transformed with pTNFtrp contained a prominent polypeptide with an apparent M_r 17,000 (Fig. 3, lanes *b*, *e*). This protein is not visible in cells transformed with pBR322 (lanes *a*, *f*), strong evidence that it represents the translational product of the TNF cDNA sequence. Furthermore, this protein co-migrates with authentic TNF (lane *c*) isolated from the HL-60 cell line (lane *d*), suggesting that no significant post-translational processing of TNF occurs in the HL-60 cell line. This is unlike lymphotoxin and γ -interferon, both of which occur naturally as heterogeneous glycoproteins as a consequence of N-terminal¹² and C-terminal³⁹ proteolysis, respectively.

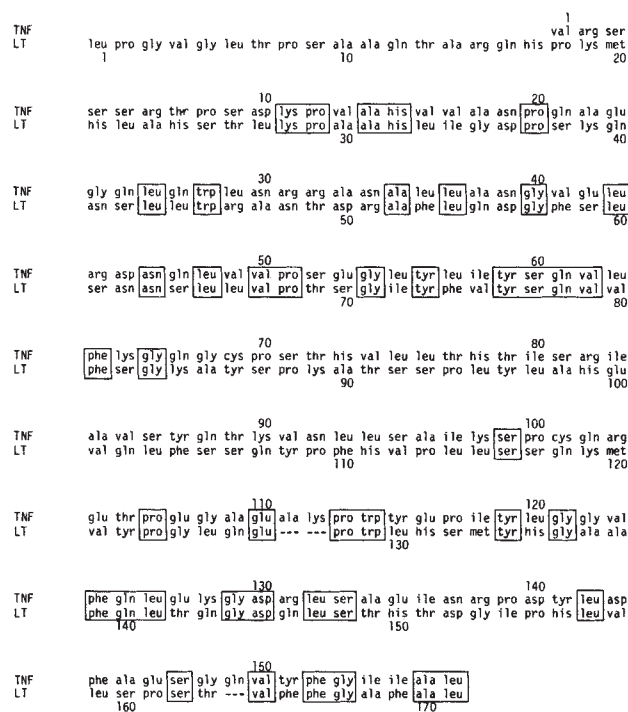


Fig. 4 Comparison of the amino acid sequence of human TNF with human lymphotoxin^{12,13}. The sequences have been aligned to give maximal homology by introducing two gaps (indicated by dashed lines) into the lymphotoxin sequence. Identical amino acids are boxed. The numbers above each row (1–157) and below each row (1–171) indicate the amino acids of mature TNF and lymphotoxin (LT), respectively.

Verification of the bacterial production of biologically-active TNF was obtained by assaying extracts of *E. coli* W3110/pTNFtrp for cytolytic activity in the murine L-929 fibroblast assay¹¹. Approximately 300,000 units of activity were detected per ml of culture at $A_{550}=1$, whereas no activity was observed in *E. coli* W3110/pBR322 controls. This corresponds to ~3 mg of active TNF per l ($A_{550}=1$) or about 300,000 molecules of active TNF per cell if a specific activity of 10^8 units mg^{-1} (ref. 16) is assumed. The activity was neutralized by antiserum prepared against partially purified PBL-derived TNF, but was not neutralized by preimmune serum or rabbit anti-human lymphotoxin antibodies (data not shown).

In vivo necrosis activity

TNF is generally defined as a cytotoxin released by BCG/LPS-treated macrophages which induces the haemorrhagic necrosis of methylcholanthrene-induced (MethA) sarcomas in BALB/c mice^{1,2}. Therefore, we examined recombinant human TNF purified from *E. coli* and natural human TNF from PMA-induced HL-60 cultures for *in vivo* tumour necrosis activity in the MethA assay¹. Both recombinant and natural TNF samples elicited significant necrotic responses, regardless of whether the TNF was injected intralesionally or systemically (Table 2). Minimal or no necrosis of the MethA sarcoma tumours was observed in mice injected with either phosphate-buffered saline (PBS) or 100 μg *E. coli* LPS. These results, taken with the antibody neutralization and Northern hybridization data, provide further evidence that the cytotoxin described here is human TNF.

Homology to lymphotoxin

The known *in vivo* and *in vitro* biological activities of TNF and lymphotoxin are very similar^{2,3,13}. TNF and lymphotoxin are now known to be antigenically distinct molecules³. It has thus become common to distinguish these two lymphokines on the basis of the cell populations responsible for their synthesis. We have compared the amino acid sequences of human TNF and

lymphotoxin to determine whether the similarities in their biological properties might be attributed to common structural features (Fig. 4). By introducing two gaps, the lymphotoxin sequence can be aligned with the TNF sequence so that distinct homologies are apparent; we find 44 of the 157 TNF residues (28%) are identical to corresponding lymphotoxin amino acids with many of the remaining differences between the two polypeptides resulting from conservative amino acid changes. The nucleotide homology over this coding region is 46% (data not shown). Two particularly conserved regions occur at amino acids 35–66 and 110–133 (TNF numbering) where 50% of the residues (28 of 56) are identical for TNF and lymphotoxin. The hydrophobic carboxy-termini of the two molecules are also significantly conserved. It is probable that the conserved regions are crucial to the shared cytotoxic activities of TNF and lymphotoxin, perhaps through interaction with a common receptor expressed on the surface of transformed cells. Support for this hypothesis is provided by the lack of cytotoxic activity in a truncated lymphotoxin polypeptide lacking its last 16 amino acids¹³.

Lymphotoxin has 18 more NH_2 -terminal amino acids than TNF (Fig. 4), suggesting that this region is not required for cytotoxic activity. In fact, a 148 residue lymphotoxin, consisting of amino acids 24–171 of mature lymphotoxin, and having similar cytotoxic effects on L-929 cells, has been isolated from the RPMI-1788 cell line^{11,12}. It is also interesting that amino acids 67–109 of TNF are unrelated to the corresponding region of lymphotoxin; only two of 43 residues are identical. This region includes all of the amino acids spanned by the Cys 69–Cys 101 disulphide bridge of TNF. One possible role for this non-conserved region could be to position correctly the two surrounding homologous regions in a conformation essential for cytotoxic activity. Such positioning, which could be achieved by a TNF disulphide bond, may require a very different sequence of amino acids in lymphotoxin, where no disulphide bond exists. These apparently unrelated regions of TNF and lymphotoxin might specify also as yet undiscovered differences in biological activities and/or target sites between the two molecules. The availability of efficient expression systems for TNF and lymphotoxin¹³, in combination with the techniques of site-directed mutagenesis⁴⁰, will make it possible to address questions of this type directly.

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1. Carswell, E. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 3666–3670 (1975).
2. Ruff, M. R. & Gifford, G. E. in *Lymphokines* Vol. 2 (ed. Pick, E.) 235–275 (Academic, New York, 1981).
3. Stone-Wolf, D. S. *et al. J. exp. Med.* **159**, 828–843 (1984).
4. Helson, L., Green, S., Carswell, E. & Old, L. J. *Nature* **258**, 731–732 (1975).
5. Matthews, N. & Watkins, J. F. *Br. J. Cancer* **38**, 302–309 (1978).
6. Matthews, N. *Immunology* **44**, 135–142 (1981).
7. Ruff, M. R. & Gifford, G. E. *Infect. Immun.* **31**, 380–385 (1981).
8. Green, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 381–385 (1976).
9. Hoffman, M. K., Oettgen, H. F., Old, L. J., Mittler, R. S. & Hammerling, U. *J. reticuloendothel. Soc.* **23**, 307–319 (1978).
10. Männel, D. N., Moore, R. N. & Mergenhagen, S. E. *Infect. Immun.* **30**, 523–530 (1980).
11. Aggarwal, B. B., Moffat, B. & Harkins, R. N. *J. biol. Chem.* **259**, 686–691 (1984).
12. Aggarwal, B. B., Henzel, W. J., Moffat, B., Kohr, W. J. & Harkins, R. N. *J. biol. Chem.* (in the press).
13. Gray, P. W. *et al. Nature* **312**, 721–724 (1984).
14. Collins, S. J., Gallo, R. C. & Gallagher, R. E. *Nature* **270**, 347–349 (1977).
15. Sundstrom, C. & Nilsson, K. *Int. J. Cancer* **17**, 565–577 (1976).
16. Aggarwal, B. B. *et al. J. biol. Chem.* (in the press).
17. Matteucci, M. & Caruthers, M. H. *J. Am. chem. Soc.* **103**, 3185–3190 (1981).
18. Grantham, R., Gautier, C., Gouy, M., Jacobzone, M. & Mercier, R. *Nucleic Acids Res.* **9**, r43–73 (1981).
19. Gray, P. W. *et al. Nature* **295**, 503–508 (1982).
20. Pennica, D. *et al. Nature* **301**, 214–221 (1983).
21. Anderson, S. & Kingston, I. B. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6838–6842 (1983).
22. Ullrich, A., Berman, C. H., Dull, T. J., Gray, A. & Lee, J. M. *EMBO J.* **3**, 361–364 (1984).
23. Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y. & Goeddel, D. V. *Cell* **38**, 287–297 (1984).

24. Jaye, M. *et al.* *Nucleic Acids Res.* **11**, 2325-2335 (1983).
25. Ullrich, A. *et al.* *Nature* **309**, 418-425 (1984).
26. Huynh, T. V., Young, R. A. & Davis, R. W. in *DNA Cloning Techniques: A Practical Approach* (ed. Glover, D.) (IRL, Oxford, in the press).
27. Proudfoot, N. J. & Brownlee, G. G. *Nature* **263**, 211-214 (1976).
28. Nissen-Meyers, J. & Hammerstrom, J. *Infect. Immun.* **38**, 67-73 (1982).
29. Kozak, M. *Nature* **308**, 241-246 (1984).
30. Blobel, G. *et al.* *Symp. Soc. exp. Biol.* **33**, 9-36 (1979).
31. Watson, M. E. *Nucleic Acids Res.* **12**, 5145-5164 (1984).
32. Hunter, E. *et al.* *J. Virol.* **46**, 920-936 (1983).
33. Nakanishi, S. *et al.* *Nature* **278**, 423-427 (1979).
34. Noda, M. *et al.* *Nature* **295**, 202-206 (1982).
35. Gubler, U. *et al.* *Nature* **295**, 206-208 (1982).
36. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. *Nature* **298**, 240-244 (1982).
37. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
38. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205 (1980).
39. Rinderknecht, E., O'Connor, B. H. & Rodriguez, H. *J. biol. Chem.* **259**, 6790-6797 (1984).
40. Zoller, M. J. & Smith, M. *Meth. Enzym.* **100**, 468-500 (1983).
41. Kumagai, K., Itoh, K., Hinuma, S. & Tapa, M. *J. immun. Meth.* **29**, 17-25 (1979).
42. Wetzel, R. *et al.* *Biochemistry* **19**, 6096-6104 (1980).
43. Aviv, H. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408-1412 (1972).
44. Wood, W. I. *et al.* *Nature* **312**, 330-337 (1984).
45. Benton, W. D. & Davis, R. W. *Science* **196**, 180-182 (1977).
46. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
47. Messing, J. & Vieira, J. *Gene* **19**, 269-276 (1982).
48. Messing, J., Crea, R. & Seeburg, P. H. *Nucleic Acids Res.* **9**, 309-321 (1981).
49. Grosjean, H. & Fiers, W. *Gene* **18**, 199-209 (1982).
50. Shepard, H. M., Yelverton, E. & Goeddel, D. V. *DNA* **1**, 125-131 (1982).
51. Gray, G. L. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 2645-2649 (1984).
52. Maizels, N. *Nature* **249**, 647 (1974).

Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing

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A novel mammalian neuropeptide, the tachykinin substance K, is specified by a discrete genomic segment. Alternative RNA splicing generates two distinct mRNAs encoding the neuropeptide substance P alone or with substance K from a single preprotachykinin gene. Relative amounts of the mRNAs vary in different tissues, suggesting that the substance K-encoding sequence is regulated in a tissue-specific manner.

SUBSTANCE P is one of the best characterized neuropeptides in mammalian tissues; several lines of evidence suggest that it acts as a neurotransmitter or neuromodulator in primary sensory neurones¹. Substance P belongs to a family of related peptides, the tachykinins, and is thought to be the only member of this family present in mammalian tissues². Recently, we elucidated the entire primary structures of two types of bovine brain substance P precursors (α - and β -preprotachykinins) by determining their cloned cDNA sequences³. β -Preprotachykinin (β -PPT) contains not only the substance P sequence but also a novel tachykinin sequence designated substance K, whereas α -preprotachykinin (α -PPT) lacks the latter sequence, containing only substance P. The decapeptide substance K has been found independently as neurokinin α , a gut-contracting peptide in porcine spinal cord⁴. The chemically synthesized substance K peptide possesses biological activities characteristic of the tachykinin family, but is considerably more potent than substance P in some pharmacological tests^{5,6}. Substance K thus represents a second type of mammalian tachykinin which may have a physiological role different from that of substance P in mammalian organisms.

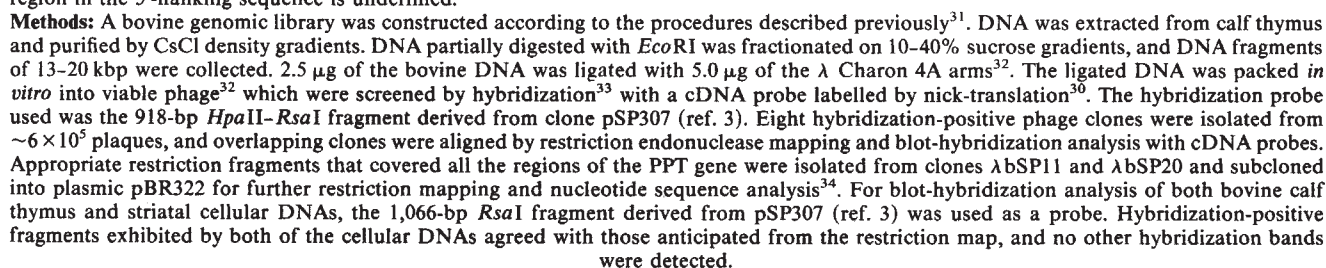
The two PPT mRNAs exhibit an interesting structural relationship. They have complete identity in their 5' and 3' sequences and differ only in the insertion/deletion of the sequence coding for the substance K region³. This characteristic structural relationship poses intriguing questions about the gene organization for these two mRNAs and the regulation for the generation of the two biologically different mammalian tachykinins. Our present investigations thus concern the structural organization of the PPT gene and the distribution and regulation of the two PPT mRNAs in the nervous system and peripheral tissues. We report here that the sequence specifying the substance K region is encoded by a discrete genomic segment, and that both α - and β -PPT mRNAs arise from a single gene by alternative RNA splicing events. We also present evidence indicating the tissue-specific regulation of the PPT gene for the differential generation of the two PPT mRNAs.

PPT gene organization

Genomic clones containing the bovine preprotachykinin gene were isolated from a bovine genomic library by hybridization

in situ with a bovine β -PPT cDNA probe, and all the isolated genomic DNA fragments were arranged into an approximately 36 kilobase-pair (kbp) length of a continuous genomic DNA (Fig. 1a; see Fig. 1 legend for experimental details of cloning). Nucleotide sequence analysis was performed on DNA fragments containing exons and their surrounding regions (Fig. 1b-f). Comparison of the genomic DNA sequence with the cDNA sequence enabled us to construct a structural organization of the bovine PPT gene (Fig. 1g). Intron A (403 base pairs, bp) is inserted within the segment encoding the 5'-untranslated region of the mRNA, 9-10 bp upstream from the translational initiation site. Introns B (~1.0 kbp), C (~450 bp), D (~460 bp), E (~1.4 kbp) and F (~3.6 kbp) all interrupt the protein-encoding region of the gene. The sequences at the exon-intron boundaries are consistent with the splice junction sequences observed for other genes⁷. Exons 2-7 consist of 132, 97, 45, 24, 54 and 596 bp, each encoding the protein sequence corresponding to the signal peptide, substance P, two spacer sequences, substance K, and the C-terminal sequence, respectively. It is remarkable that exon 6 precisely specifies the substance K region missing in α -PPT. Because blot-hybridization analysis of total cellular DNAs (data not shown) as well as the genomic cloning described above showed that no more than one PPT gene is present in the bovine genome, we conclude that both α - and β -PPT mRNAs are produced from a single gene as a consequence of alternative RNA splicing events.

The 5' termini of the PPT mRNAs were identified by S₁ nuclease mapping and primer extension analyses (Fig. 2). Both analyses revealed a length heterogeneity at the 5' end of the PPT transcripts. The major 5' termini of the PPT mRNAs mapped at 106-108, 110 and 111 bp upstream from the 3' end of exon 1 (Fig. 1g). Several minor mRNA species starting further upstream were also observed and these 5' termini mapped at roughly 132, 133, 137 and 146 bp upstream from the 3' end of exon 1. In support of these assignments, we found that three of the four cDNA clones isolated previously³ (clones pSP301, 302 and 306) contained the extreme 5' sequences corresponding to the major 5' ends, while the remaining one (clone pSP307) extended its 5'-terminus up to one of the minor 5' ends. Based on the assignments of the 5' termini of the PPT mRNAs, we conclude that the bovine PPT gene is ~8.4 kbp long.



major transcription initiation sites. An A residue is generally preferred as a capping site for transcription initiation⁷. Therefore, a microheterogeneity of the major 5' ends of the PPT mRNAs may reflect an imprecision in the polymerase nucleotide selection mechanism and the possible use of the multiple capping sites.

Table 1 Relative levels of PPT mRNAs and relative amounts of α - and β -PPT mRNAs in bovine nervous system and peripheral tissues

Region or tissue assayed	PPT mRNAs	β -PPT mRNA/ α -PPT mRNA
Trigeminal ganglion	100	0.65 (0.49)
Caudate nucleus	70	0.29 (0.31)
Putamen	42	0.29 (0.33)
Olfactory bulb	32	0.83 (0.48)
Midbrain	20	0.28 (0.28)
Hypothalamus	9	0.31 (0.33)
Amygdala	6	0.38 (0.30)
Medulla oblongata	5	0.26 (0.24)
Thalamus	2	ND (0.30)
Pons	<1	ND (0.28)
Thyroid	2	>30 (>30)
Duodenum	2	9 (5)
Small intestine	1	7 (4)

The relative levels of PPT mRNAs in the brain region and peripheral tissues (left-hand column) are expressed as per cent of that in the trigeminal ganglion. Besides the above tissues, a little of the PPT mRNAs was identified in the adrenal medulla and none of the PPT mRNAs were detected in the bovine kidney, liver, urinary bladder or submaxillary gland. The ratios of β -PPT mRNA to α -PPT mRNA measured with 3'-end-labelled cDNA probe are presented in the right-hand column; values in parentheses indicate results obtained with 5'-end-labelled cDNA probe. ND, not determined because of faint bands. The relative levels of PPT mRNAs and the relative amounts of α - and β -PPT mRNAs were quantitated by densitometric scan analysis of autoradiographs of RNA blot-hybridization and S_1 nuclease protection analyses respectively. The RNA blot-hybridization analysis was performed as described previously²⁸ using total RNA (40 μ g) extracted²⁹ from various bovine brain regions and peripheral tissues; the hybridization probe used ($\sim 1.0 \times 10^5$ c.p.m. μ g⁻¹, 10 ng ml⁻¹) was the 1,066-bp *Rsa*I fragment derived from pSP307 (ref. 3) and labelled by nick-translation³⁰. The S_1 nuclease protection assays were carried out as described for Fig. 4. In both RNA blot-hybridization and S_1 nuclease protection analyses, the densitometric scan analysis was adjusted for the time of exposure such that each band analysed was within the linear portion of the exposure. The values given are based on multiple independent experiments.

Distribution of PPT mRNAs

To investigate the tissue distribution of bovine PPT mRNAs and their regional distribution in the brain, RNAs isolated from various tissues and brain regions were analysed by blot-hybridization techniques. Because α - and β -PPT mRNAs did not separate on agarose gel electrophoresis (data not shown), the sum of the PPT mRNA levels were quantified by scanning densitometry of autoradiographic exposures of the blot-hybridization analysis (Table 1, left column).

Previous studies combining surgical manipulations with immunohistochemistry identified various substance P-projecting neuronal pathways in the central and peripheral nervous system⁸. Figure 3 illustrates schematically the relationship between the major substance P-projecting pathways in the brain and the regional distribution of the PPT mRNAs determined in the present study. We find good correlation between the regions or nuclei that exhibit high levels of PPT mRNAs and those projecting the substance P-containing fibres. Because these regions express α -PPT mRNA over β -PPT mRNA, the substance P immunoreactivity identified in these regions may reflect mainly the peptide derived from α -PPT. It is thus possible that some of the substance K peptide derived from β -PPT may be transported to different nerve terminals as a neurotransmitter or neuromodulator for a different neuronal communication.

Expression of the PPT gene

To examine possible differences in the expression of the two mRNAs in the central nervous system (CNS) and in peripheral tissues, we next investigated the relative amounts of bovine α - and β -PPT mRNAs in various tissues and in various brain regions by S_1 nuclease protection analysis. Two cDNA probes were used for this analysis (Fig. 4c, d), and the results obtained

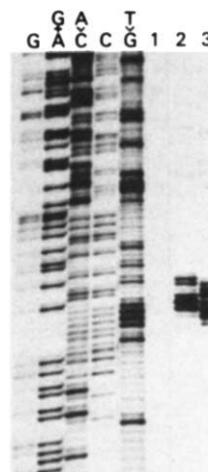


Fig. 2 Identification of the 5' termini of bovine PPT mRNAs by S_1 mapping and primer extension analyses. An autoradiograph shows the chemically degraded products used as a chain length marker (five left lanes), the S_1 digestion products using yeast tRNA (lane 1) and striatal poly(A) RNA (lane 2) and the primer-extended cDNAs using striatal poly(A) RNA (lane 3).

Methods: Poly(A) RNA was isolated from bovine striata³. The *Nci*I fragment containing most of the exon 1 sequence and its 5'-flanking sequence (Fig. 1b) was isolated from the 2.6-kbp *Bam*HI fragment containing exons 1 and 2, labelled with ³²P at the 5' ends and cleaved by *Hae*III or *Fnu*4HI. The 332-bp *Nci*I-*Hae*III fragment (15 fmol, 1.0×10^5 c.p.m.) and the 38-bp *Nci*I-*Fnu*4HI fragment (15 fmol, 1.0×10^5 c.p.m.) were used for S_1 mapping³⁵ and primer extension analyses³⁶, respectively; both DNAs were labelled at the same *Nci*I site present 17 nucleotides upstream from the 3' end of exon 1 (Fig. 1g). The S_1 digestion products and the primer-extended cDNAs were electrophoresed parallel to chemically degraded products of the 332-bp *Nci*I-*Hae*III fragment on 8.3 M urea/8% polyacrylamide gel.

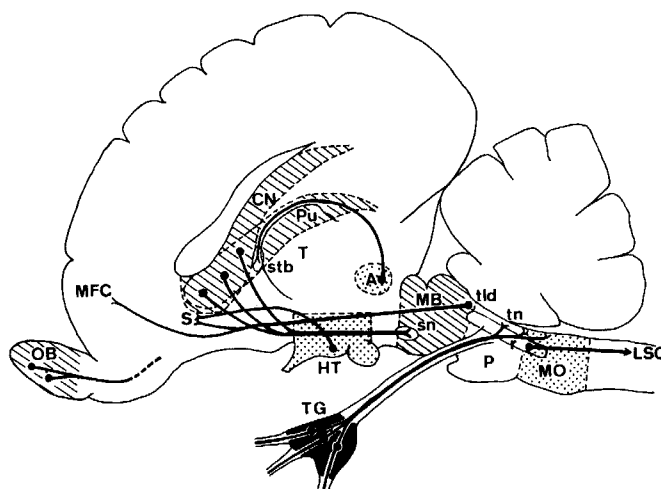
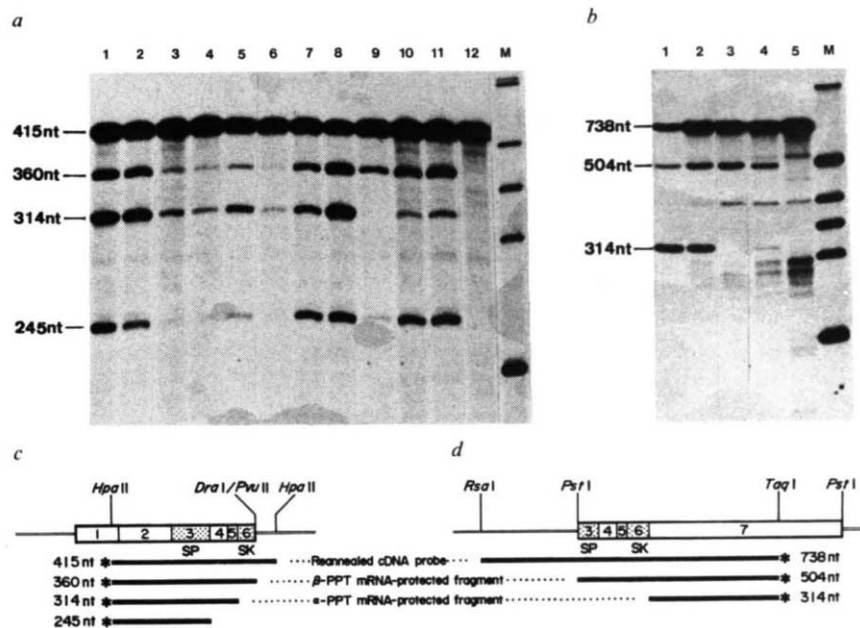


Fig. 3 The relationship between the regional distribution of PPT mRNAs and the major substance P-projecting pathways. According to the relative levels of PPT mRNAs in various brain regions presented in Table 1 (left column), the regions which exhibit high levels of PPT mRNAs are classified into three groups: black area, 100 (the trigeminal ganglion); shaded area, 20-70; dotted area, 5-9. The major substance P-projecting pathways are based on data reviewed in ref. 8: TG, trigeminal ganglion; OB, olfactory bulb; MFC, medial frontal cortex; CN, caudate nucleus; Pu, putamen; T, thalamus; A, amygdala; HT, hypothalamus; MB, midbrain; P, pons; MO, medulla oblongata; stb, bed nucleus of stria terminalis; S, lateral septal area; sn, substantia nigra; tld, nucleus laterodorsalis tegmenti; tn, spinal nucleus of trigeminal nerve; r, raphe nuclei; LSC, lumbar spinal cord.

Fig. 4 S_1 nuclease protection analysis of PPT mRNAs. *a, b*, Autoradiographs of *c, d*; *c, d*, diagrams of the S_1 nuclease-resistant DNA fragments (thick lines) in the protection assays using 3'- and 5'-end-labelled cDNA probes. *a*, RNAs analysed as follows: lane 1, caudate nucleus; lane 2, putamen; lane 3, hypothalamus; lane 4, amygdala; lane 5, mid-brain; lane 6, medulla oblongata; lane 7, olfactory bulb; lane 8, trigeminal ganglion; lane 9, thyroid; lane 10, duodenum; lane 11, small intestine; lane 12, liver (control). M, 32 P-labelled markers of *HinfI*-digested pBR322 DNA. *b*, The following RNAs were used: lane 1, hypothalamus; lane 2, trigeminal ganglion; lane 3, thyroid; lane 4, small intestine; lane 5, liver (control). M, markers. *c, d*, Open boxes and double lines at the top indicate the cDNA sequences and their flanking vector sequences, respectively; the numbered boxes indicate the corresponding exon sequences, and the dotted boxes encode the substance P (SP) substance K (SK) sequence. The asterisks denote the 32 P-labelled sites. nt, Nucleotides.

Methods: *a*, The ~750-bp *HindIII*-*DraI* fragment containing a portion of the β -PPT cDNA sequence and its flanking vector sequence was isolated from clone pSP302 (ref. 3) and subcloned into pBR322 at the *HindIII* and *PvuII* sites (clone pPPT $_{\beta 2}$). pPPT $_{\beta 2}$ DNA was digested with *HpaII* and was 32 P-labelled at the 3' ends with [α - 32 P] dCTP. The 415-bp *HpaII* fragment was isolated and used as a probe. This probe contained the nucleotide sequence complementary to 360 nucleotides of β -PPT mRNA and 55 nucleotides of the vector sequence. The probe (6.3 fmol, 4.0×10^4 c.p.m.) was denatured, hybridized to total RNA (40 μ g, lanes 1–8, 12) or poly(A) RNA (40 μ g, lanes 9–11) and digested with S_1 nuclease (10 U per μ g RNA). *b*, The 661-bp *PstI* fragment containing a portion of the β -PPT cDNA sequence was isolated from clone pSP307 (ref. 3) and subcloned into pBR322 (clone pPPT $_{\beta 1}$). pPPT $_{\beta 1}$ DNA was digested with *TaqI*, 32 P-labelled at the 5' ends and cleaved by *RsaI*. The 738-bp *TaqI*-*RsaI* fragment used as a probe contained the nucleotide sequence complementary to 504 nucleotides of β -PPT mRNA and 234 nucleotides of the vector sequence. The probe (17 fmol, 1×10^5 c.p.m.) was denatured, hybridized to poly(A) RNA (10 μ g) and digested with S_1 nuclease (10 U per μ g RNA). The S_1 digestion products were electrophoresed parallel to the markers on 8.3 M urea/8% polyacrylamide gel. Exposure time for lanes 3–5 is three times longer than that for lanes 1–2 in *b*.



with use of the 3'- and 5'-end-labelled cDNA probes are presented in Fig. 4*a* and *b*, respectively. The analysis with the former cDNA probe gave rise to three RNA-protected bands with estimated sizes of 360, 314 and 245 nucleotides. The former two sizes corresponded to those representing the cDNA fragments protected by β - and α -PPT mRNAs, respectively. The appearance of the 245-nucleotide fragment on this S_1 nuclease analysis, however, was unexpected. The size of this fragment corresponded to the distance between the 3'-end-labelled *HpaII* site and the 3' end of exon 3. This coincidence suggests that splicing at the 3' end of exon 3 may occur in the expression of the PPT gene, and that this different mode of RNA splicing may generate a third PPT mRNA. The analysis using the 5'-end-labelled cDNA probe, however, revealed two and not three RNA-protected bands, and the sizes of these two bands (504 and 314 nucleotides) corresponded to those representing the cDNA fragments protected by β - and α -PPT mRNAs, respectively. If the third PPT mRNA indeed exists in various tissues, the absence of the corresponding protection band on the second S_1 nuclease analysis may imply that there is an unidentified acceptor splice site for the formation of the third PPT mRNA. Another possible explanation is that the S_1 nuclease-resistant fragment protected by the third PPT mRNA by chance overlaps with the ~380 nucleotide band observed in the control lane. If so, the size of this fragment suggests that the acceptor splice site for the formation of the third PPT mRNA is located at the 5' end of exon 5. However, further characterization of the possible third PPT mRNA remains to be determined.

Densitometric scannings of the autoradiographs of the S_1 nuclease protection analyses enabled us to quantitate the relative amounts of α - and β -PPT mRNAs in the bovine nervous system and peripheral tissues irrespectively of the third PPT mRNA (Table 1, right column). There is a good agreement between the values obtained with the two different probes. Table 1 shows clearly that there are marked variations in the relative amounts of α - and β -PPT mRNAs among the various tissues tested. The level of α -PPT mRNA is two to three times higher than that of

β -PPT mRNA in the nervous system. In peripheral tissues, this relation of the two mRNAs is completely reversed and the level of β -PPT mRNA is considerably higher than that of α -PPT mRNA. This is particularly remarkable in the thyroid gland where virtually no α -PPT mRNA was detected (Fig. 4*a*, lane 9; *b*, lane 3). The observed tissue-specific variations in the relative amounts of the two mRNAs are independent of the levels of the PPT mRNAs (Table 1). The CNS exhibits an almost constant ratio of the two mRNAs in spite of the marked differences in the levels of the PPT mRNAs, with the exception of the olfactory bulb. We therefore conclude that the expression of the two PPT mRNAs is regulated in a tissue-specific manner via the specific inclusion or exclusion of a peptide-coding sequence by means of different RNA splicing events.

Figure 5 schematically illustrates the expression of the two PPT mRNAs. α -PPT mRNA is mainly synthesized in the nervous system and codes for the substance P-containing precursor, whereas β -PPT mRNA is produced in both the nervous system and some peripheral tissues and codes for the substance P- and substance K-containing precursor. The two mammalian tachykinins substance P and substance K exhibit markedly different biological activities on a wide range of mammalian tissues^{5,6}; such differences can be ascribed to the existence of at least two types of the tachykinin receptors, termed SP-P and SP-E receptors^{5,9}. The two receptors are supposed to have different affinities for substance P and substance K and to be distributed differently in various mammalian tissues. Thus, through the selectivity of the two tachykinin receptors, the differential expression of the two mRNAs from the single PPT gene would ultimately produce different physiological responses in the CNS as well as in peripheral tissues.

Conclusions

Examples of alternative RNA splicing for the generation of several mRNAs from a single gene have been reported for some viral systems^{10,11} and cellular genes^{12–21}. Apart from viral genes, examples of sequence differences between mRNAs from a single

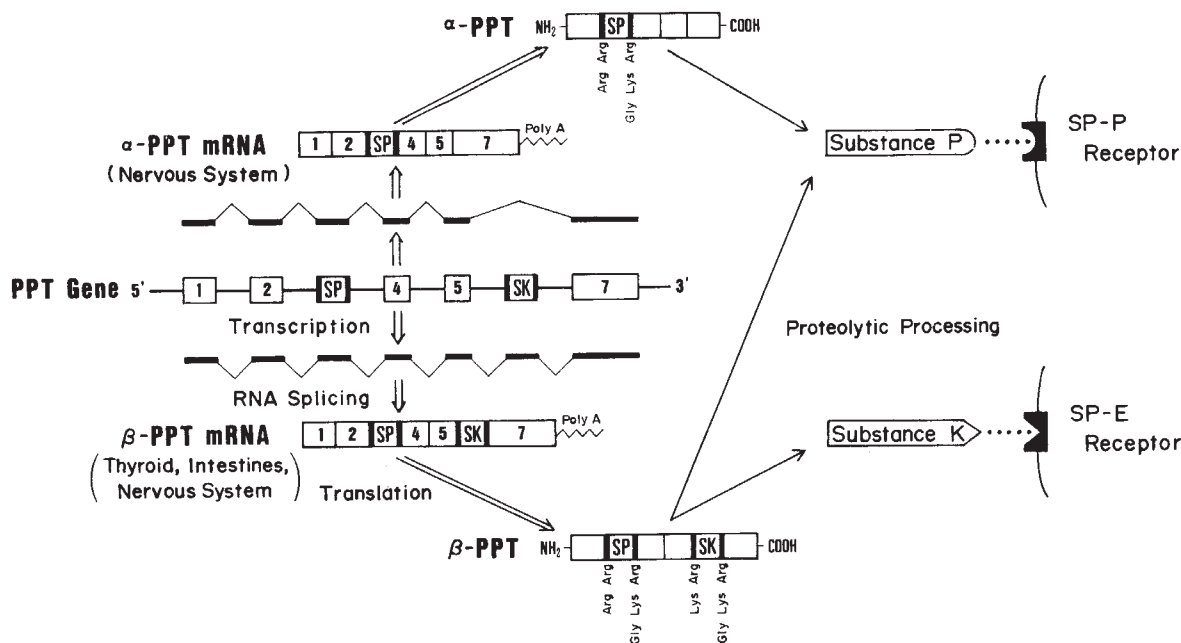


Fig. 5 Schematic representation of alternative RNA splicing pathways in the expression of the PPT gene. In the structure of the PPT gene, numbered boxes and those indicated as SP and SK stand for the corresponding exons and the exons encoding substance P (exon 3) and substance K (exon 6), respectively. Tissues in which β -PPT mRNA is predominantly expressed or both α - and β -PPT mRNAs are expressed are shown in parentheses. Amino acids under the protein structures indicate those involved in the proteolytic processing and the C-terminal amidation for the formation of substance P and substance K.

gene predominantly concern differences in the 5' and 3' ends of the mRNAs¹²⁻¹⁷. In these cases, the generation of different mRNAs could result from the use of alternative promoters or polyadenylation sites. In contrast, the PPT gene represents a novel example in which differential splicing events seem to be independent of alternative promoters or polyadenylations. The inclusion or exclusion of a separated exon sequence by alternative splicing, as described here, has been reported for the *Drosophila* myosin gene²⁰ and the murine α A-crystallin gene²¹. In the previously reported cases, however, it has not been clear whether alternative splicing events serve any functions. The PPT gene thus provides an intriguing system in which the inclusion or exclusion of a whole exon sequence by alternative RNA splicing events leads to the production of functionally different polypeptides.

The structure of the PPT gene and its RNA products strongly suggest that the true regulation of splicing choice takes place in the PPT primary transcript and that this molecular mechanism is responsible for the observed tissue-specific alteration in the expression of the two PPT mRNAs. Such splicing regulation could arise from changes in enzyme specificity, small nuclear RNAs and other factors affecting the splicing process. The change of the chromatin structure or the modification of the gene structure may also affect the accessibility of the splicing machinery to a splice site. Another mechanism could involve the transcription initiation at different sites affecting a splice site of the PPT primary transcripts. This possibility seems unlikely because we identified the same multiple transcription initiation sites for both α - and β -PPT mRNAs on S₁ nuclease mapping analysis (data not shown). A further possibility is that although all the nucleotide sequences of the exon-intron junctions of the PPT gene agree with the consensus sequence found at the exon-intron boundaries of other genes⁷ (Fig. 1g), a small rearrangement of the gene structure or nucleotide changes in the gene may occur and change a splice site of the primary transcript.

The observed tissue-specific variations in the relative amounts of the two PPT mRNAs raise interesting questions about what types of cells express PPT mRNAs as well as whether there is cell-type-specific regulation of their expression. It was shown

previously that enterochromaffin cells are the main substance P-immunoreactive cells in the intestines²². Furthermore, the medullary carcinoma of the thyroid was found to contain large amounts of substance P²³. Thus, enterochromaffin cells and thyroid C cells may involve the predominant expression of β -PPT mRNA in the intestines and thyroid. Similarly, a particular neurone may express either α - or β -PPT mRNA throughout its lifetime, implying the presence of separate groups of neurones containing substance P alone or both substance P and substance K. Alternatively, a given neurone may be able to switch its pattern of PPT mRNA production during development or in response to external stimuli. These questions are important for further understanding of the physiological role of the mammalian tachykinins.

Specific neurones or sets of neurones may use a variety of peptides to communicate with one another and to express an invariant programme during development. Such informational ability may have been expanded during evolution by the increase of the diversity of neuropeptides in several ways. These include gene duplication^{24,25}, repetition of multiple neuropeptides within a single precursor²⁵⁻²⁸ and alternative RNA processing in combination with different polyadenylations and RNA splicing¹⁵. We have presented here another novel example in which the diversity of neuropeptides can be increased by the specific inclusion and exclusion of the internally separated exon sequence encoding the neuropeptide. The characteristic feature of this system is that the regulation of the inclusion/exclusion of the peptide-coding sequence would ultimately change the ratio of the combination of the biologically different peptides. In addition, this system can simultaneously provide a means for the coordinate synthesis of the different peptides under the control of a single promoter, once the peptides are encoded in a single transcription unit. The PPT gene thus represents an interesting model system not only for exploring mechanisms responsible for the tissue-specific regulation in the expression of the eukaryotic gene but also for understanding the molecular basis for the generation of the diversity of neuropeptides in the neuroendocrine system.

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1. Otsuka, M. & Konishi, S. *Cold Spring Harb. Symp. quant. Biol.* **40**, 135-143 (1975).
2. Erspamer, V. *Trends Neurosci.* **4**, 267-269 (1981).
3. Nawa, H., Hirose, T., Takashima, H., Inayama, S. & Nakanishi, S. *Nature* **306**, 32-36 (1983).
4. Kimura, S., Okada, M., Sugita, Y., Kanazawa, I. & Munekata, E. *Proc. Jap. Acad.* **B59**, 101-104 (1983).
5. Nawa, H., Doteuchi, M., Igano, K., Inouye, K. & Nakanishi, S. *Life Sci.* **34**, 1153-1160 (1984).
6. Hunter, J. C. & Maggio, J. E. *Eur. J. Pharmacol.* **97**, 159-160 (1984).
7. Breathnach, R. & Chambon, P. A. *Rev. Biochem.* **50**, 349-383 (1981).
8. Cuervo, A. C., Priestley, J. V. & Matthews, M. R. *Ciba Fdn Symp.* **91**, 55-83 (1982).
9. Iversen, L. L. *et al. Ciba Fdn Symp.* **91**, 186-205 (1982).
10. Ziff, E. B. *Nature* **287**, 491-499 (1980).
11. Nevins, J. R. *Cell* **28**, 1-2 (1982).
12. Alt, F. W. *et al. Cell* **20**, 293-301 (1980).
13. Early, P. *et al. Cell* **20**, 313-319 (1980).
14. Maki, R. *et al. Cell* **24**, 353-365 (1981).
15. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. *Nature* **298**, 240-244 (1982).
16. Young, R. A., Hagenbüchle, O. & Schibler, U. *Cell* **23**, 451-458 (1981).
17. Kitamura, N. *et al. Nature* **305**, 545-549 (1983).

18. DeNoto, F. M., Moore, D. D. & Goodman, H. M. *Nucleic Acids Res.* **9**, 3719-3730 (1981).
19. Schwarzbauer, J. E., Tamkun, J. W., Lemischka, I. R. & Hynes, R. O. *Cell* **35**, 421-431 (1983).
20. Rozek, C. E. & Davidson, N. *Cell* **32**, 23-34 (1983).
21. King, C. R. & Piatigorsky, J. *Cell* **32**, 707-712 (1983).
22. Nilsson, G. *et al. Histochemistry* **43**, 97-99 (1975).
23. Skrabanek, P. *et al. Experientia* **35**, 1259-1260 (1979).
24. Blundell, T. L. & Humbel, R. E. *Nature* **287**, 781-787 (1980).
25. Scheller, R. H. *et al. Cell* **32**, 7-22 (1983).
26. Nakanishi, S. *et al. Nature* **278**, 423-427 (1979).
27. Noda, M. *et al. Nature* **295**, 202-206 (1982).
28. Kakidani, H. *et al. Nature* **298**, 245-249 (1982).
29. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294-5299 (1979).
30. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237-251 (1977).
31. Maniatis, T. *et al. Cell* **15**, 687-701 (1978).
32. Blattner, F. R. *et al. Science* **196**, 161-169 (1977).
33. Benton, W. D. & Davis, R. W. *Science* **196**, 180-182 (1977).
34. Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499-560 (1980).
35. Berk, A. J. & Sharp, P. A. *Cell* **12**, 721-732 (1977).
36. Ghosh, P. K., Reddy, V. B., Piatka, M., Lebowitz, P. & Weissman, S. M. *Meth. Enzym.* **65**, 580-595 (1980).

LETTERS TO NATURE

Nuclearites—a novel form of cosmic radiation

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E. Witten (personal communication) has raised the intriguing possibility that nuclear matter consisting of aggregates of up, down and strange quarks in roughly equal proportions may be less massive than ordinary nuclear matter of the same quark number consisting of protons and neutrons (triplets of non-strange quarks). These nuggets of strange quark matter may be stable for almost any baryon number (A), including values intermediate between those of ordinary nuclei ($A \leq 263$) and neutron stars ($A \sim 10^{57}$). We use the term 'nuclearite' to describe such strange quark nuggets in collision with Earth and suggest experiments to detect these encounters.

Ordinary nuclear matter has an energy density of ~ 938 MeV per nucleon. Ordinary quark matter—a purely hypothetical form of matter in which the constituent up and down quarks and gluons are not confined within individual nucleons—evidently has a higher energy density, for otherwise nuclei would decay into quark matter. Strange quark matter (consisting of equal numbers of up, down and strange quarks) differs energetically from ordinary quark matter in two ways: strange quarks are considerably heavier than ordinary quarks, which is why strange baryons are some 200 MeV heavier than nucleons. On the other hand, the Pauli exclusion principle acts so as to make strange quark matter more stable than ordinary quark matter. In a degenerate gas of massless quarks at zero temperature, the energy per quark depends on the number of quark species, and in a three-flavour system (strange quark matter) it is estimated by Witten to be $\sim 90\%$ of what it is in a two-flavour system. This additional (negative) symmetry energy may more than compensate the positive energy penalty associated with the strange quark mass.

Farhi and Jaffe¹ have performed semiquantitative calculations on the basis of the Massachusetts Institute of Technology bag model. They find that single nuggets of strange quark matter are stable for a range of strange quark masses and strong quantum chromodynamic (QCD) coupling constants in accordance with phenomenological estimates of these parameters extracted from hadron spectroscopy. In short, they are unable to decide definitely whether strange quark matter is stable or not. We may not conclude from the stability of nuclei that a stabler form of nuclear matter does not exist. The transition of, say, ^{56}Fe into strange quark matter containing 168 up, down

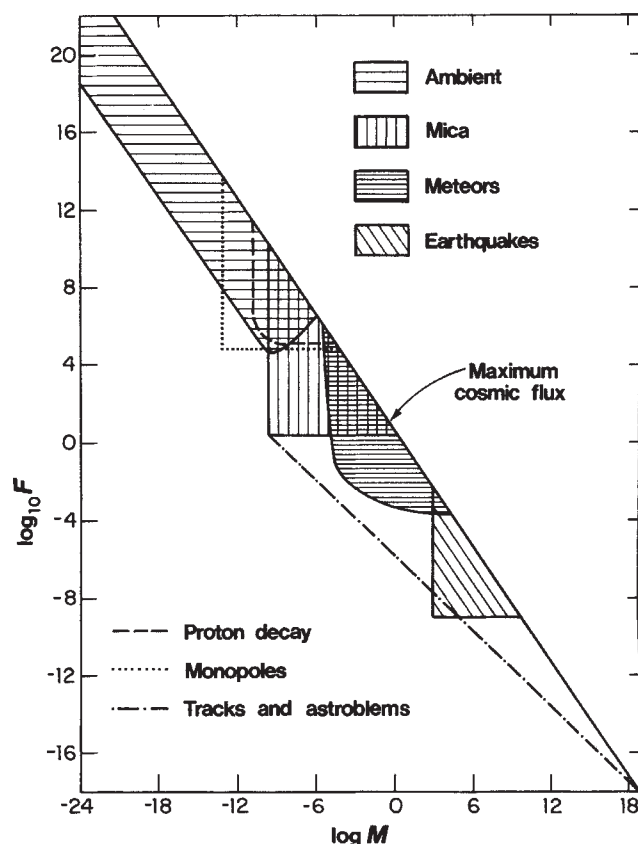


Fig. 1 The maximum possible cosmic flux of nuclearites F ($\text{km}^{-2} \text{yr}^{-1} (2\pi \text{sr})^{-1}$) as a function of mass. Also shown are regions that would be excluded by the several experiments discussed in the text. (Of these, only the 'mica' limit corresponds to an experiment actually performed.)

and strange quarks, in equal proportions, is a 56th-order weak interaction. Even if strange quark matter were stable, the lifetime of ordinary matter would be essentially infinite on cosmological time scales.

Witten offers a tentative view of the early Universe in which a large fraction of the mass and baryon number of the Universe has survived in the form of nuggets of strange quark matter (nuclearites) with radii R , between 0.1 and 10 cm, appearing shortly after the Big Bang in the QCD transition from a quasi-free quark plasma to a cooler state where quarks are confined. Strange quark matter is nonluminous (not the stuff of ordinary stars) and did not participate in primordial nucleosynthesis. Primordial nuclearites may constitute the dark missing mass of the Universe and of the Milky Way.

There may be more recent sources. What we regard as neutron stars may in fact consist of strange quark matter². Collisions

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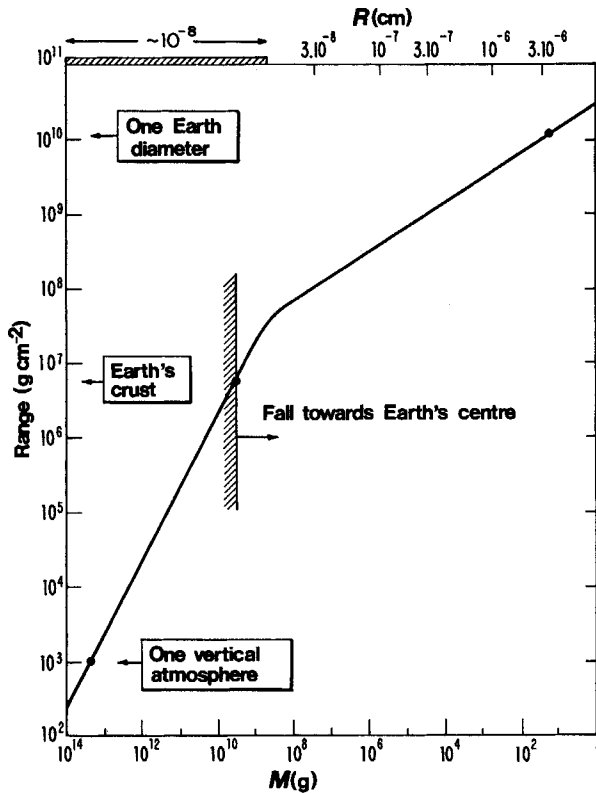


Fig. 2 The range of nuclearites as a function of their mass, as predicted by equation (5).

involving these stars, or supernovae themselves, may liberate pieces of strange quark matter with a spectrum of sizes that is difficult to guess. Nuclearites, or other unsuspected states of nuclear matter, may exist in the Galaxy as remnants of the Big Bang or as debris of astrophysical catastrophes.

Galaxies, our own included, contain significant non-luminous mass², possibly in the form of nuclearites. The local density of dark matter is estimated to be $\sim 10^{-24}$ g cm⁻³. With chaotic velocities ~ 250 km s⁻¹ characteristic of the Sun's galactic rotation, the local flux of dark matter (an upper bound to the nuclearite mass flux) is $\sim 2.5 \times 10^{-17}$ g cm⁻² s⁻¹ and the annual Earth infall is $\sim 10^9$ g. We assume that the entire flux consists only of nuclearites so as to set minimum useful sensitivities on experiments designed to discover cosmic nuclearites. If the mean mass of a cosmic nuclearite is M , the maximal cosmic flux is given by

$$F = 7.8(1 \text{ g}/M) \text{ nuclearites km}^{-2} \text{ yr}^{-1} (2\pi \text{ sr})^{-1} \quad (1)$$

Equation (1) is plotted in Fig. 1 along with regions excludable by experiments we shall consider.

The density of strange quark matter is estimated² to be $\rho_N = 3.6 \times 10^{14}$ g cm⁻³, somewhat larger than that of atomic nuclei or neutron stars. Because strange quarks are heavier than the others and so disfavoured, the net-quark charge is positive and is compensated by electrons (negative charge). Nuclearites, small compared with the Bohr radius, resemble superheavy nuclear atoms with $A < 10^{15}$ and Z well beyond any published in a periodic table. The electron distribution of larger nuclearites extends beyond the nuclear boundary by at least an electron-Compton wavelength. Nuclearites with galactic velocities are protected by their electron 'atmosphere' and coulomb repulsion from direct nuclear interactions with the atoms they may hit; the same is true of nuclearites that have come to rest in matter.

The principal energy-loss mechanism for a nuclearite passing through matter is that of atomic collisions. As it traverses the medium, it displaces all the matter in its path by elastic or quasi-elastic collisions with the ambient atoms. For a massive

nuclearite, as for meteorites⁴, the rate of energy loss is

$$\frac{dE}{dx} = -A\rho v^2 \quad (2)$$

where A is the effective cross-sectional area of the nuclearite, v is its velocity, and ρ is the density of the medium. Thus, v decreases exponentially with distance L according to

$$v(L) = v(0) \exp \left\{ -\frac{A}{M} \int_0^L \rho dx \right\} \quad (3)$$

where M is the mass of the nuclearite. In the case of a nuclearite larger than an atom ($R_0 \geq 1$ Å), its cross-sectional area is simply πR_0^2 . On the other hand, the effective area of a smaller nuclearite is controlled by its electronic atmosphere which is never smaller than ~ 1 Å. Thus, we take

$$A = \begin{cases} \pi(3M/4\pi\rho_N)^{2/3} & M \geq 1.5 \text{ ng}, \\ \pi \times 10^{-16} \text{ cm}^2 & M < 1.5 \text{ ng}. \end{cases} \quad (4)$$

Equation (2) breaks down at low velocity when the retarding force does not vanish but becomes equal (in a solid) to the force by which the confining material resists interpenetration. The integrity of rock persists up to pressures of 1,000 atm, corresponding to a structural energy density $\epsilon \approx 10^9$ erg cm⁻³, or ≈ 0.1 eV per molecular bond. For subsonic velocities, $v(L) < v_c = \sqrt{\epsilon/\rho}$, the right hand side of equation (2) must be replaced by the constant force $-\epsilon A$, and from this point the nuclearite is rather quickly brought to rest.

From equation (2), and the subsequent discussion, we compute the range of a nuclearite as a function of its mass

$$\begin{aligned} \int_0^L \rho dx &= (M/A) \ln(v(0)/v_c) \\ &= \begin{cases} 3 \times 10^7 [M/1 \text{ ng}]^{1/3} \text{ g cm}^{-2} & M \geq 1.5 \text{ ng} \\ 2.3 \times 10^7 [M/1 \text{ ng}] \text{ g cm}^{-2} & M < 1.5 \text{ ng} \end{cases} \end{aligned} \quad (5)$$

From Fig. 2, we see that nuclearites heavier than 4×10^{-14} g penetrate the atmosphere while maintaining cosmic velocities and those heavier than 0.1 g pass freely through an Earth diameter whose column density (see, for example, ref. 5) is 1.1×10^{10} g cm⁻².

Cosmic nuclearites of low-enough mass will come to rest in the Earth's crust and accumulate therein. The effect of gravity cannot be neglected: if the Earth's pull, Mg , exceeds the maximum static retarding force, ϵA , a nuclearite cannot come to rest, but will fall through the Earth. Nuclearites lighter than 0.3 ng will stop in the crust and can have developed an observable concentration. The maximal cosmic nuclearite flux given by equation (1), over the course of the Earth's 4.6-Gyr history will have produced a crustal concentration of 10^{-7} by mass. Searches for these ambient nuclearites require judicious choice of materials (deep sea sediments, excreta, ...), methods of concentration (distillation, centrifugation) and detection techniques (proton X-ray activation, mass spectroscopy, ...). The masses of accumulated nuclearites can range from those of atomic nuclei to the upper limit of 0.3 ng or $A \sim 2 \times 10^{14}$. A complete search probably requires the use of a variety of experimental approaches. Figure 1 shows the excluded region that would result from a negative search for crustal nuclearites to a concentration of 10^{-13} , a feasible experiment not yet performed.

Cosmic nuclearites, in traversing a transparent medium like water or air will deposit some of their energy in the form of visible light. The fraction of dissipated energy appearing as light is called the luminous efficiency η . A lower bound on η can be deduced from simple thermodynamical arguments in which light is emitted as black-body radiation from an expanding cylindrical thermal shock wave. The effective temperature of the shock depends on its radius, R , according to

$$T(t) = \frac{mv^2}{n} (R_0/R(t))^2 \quad (6)$$

where m is molecular mass of the medium and n is the effective

number of submolecular species that are excited. For this calculation, we use natural units where $\hbar = c = k = 1$. The rate of expansion of the shock is given by

$$\dot{R}(t) = \sqrt{\frac{2Tn}{m}} \quad (7)$$

characteristic of molecular velocities. From equations (6) and (7), we deduce

$$\begin{aligned} R^2(t) &= (8)^{1/2} vt R_0 \\ T(t) &= (8)^{-1/2} mv R_0 / nt \end{aligned} \quad (8)$$

for the time evolution of the radius and the temperature of the shock, respectively. The integration constant is chosen in such a fashion that the passage of the nuclearite takes place at the time $t_0 = R_0 / v\sqrt{8}$. The thermodynamic description of the event is valid when R exceeds the mean free path l in the medium, corresponding to the time $t_1 = (l/R_0)^2 t_0$.

The expanding hot cylinder emits black-body radiation with a spectrum

$$-\frac{dp}{d\omega da} = \frac{\omega^3}{(2\pi)^2} \frac{1}{e^{\omega/T} - 1} \quad (9)$$

of power (p) radiated per unit frequency (ω) and per unit area (a). The total luminous energy radiated by the nuclearite per unit length of its trajectory is

$$\frac{dE_\gamma}{dx} = \int_{\omega_{\min}}^{\omega_{\max}} d\omega \int_{t_{\min}}^{\infty} dt 2\pi R(t) \frac{dp}{d\omega da} \quad (10)$$

The limits on frequency correspond to the window of transparency of the medium, and $t_{\min}$ is the greater of t_0 and t_1 . For a wide transparency window and under circumstances such that

$$T(l) \gg \omega_{\max} \quad (11)$$

—a condition that is satisfied for the cases we consider—equation (10) may be approximated by

$$\frac{dE_\gamma}{dx} \approx (6\pi^2\sqrt{2})^{-1} \omega_{\max}^{5/2} (m/n)^{3/2} \pi R_0^2 v^2 \quad (12)$$

The luminous efficiency is obtained by comparing equations (2) and (12),

$$\begin{aligned} \eta &\approx (6\pi^2\sqrt{2})^{-1} \omega_{\max}^{5/2} (m/n)^{3/2} \rho^{-1} \\ &\approx 2 \times 10^{-5} (\hbar\omega_{\max}/(\pi \text{ eV}))^{5/2} (\text{MW}/18)^{3/2} (3/n)^{3/2} (\rho_w/\rho) \end{aligned} \quad (13)$$

where $\pi \text{ eV}$ is a typical visible photon energy, MW is the mean molecular weight of the medium, $n=3$ is an estimate of the relevant number of excited subnuclear degrees of freedom and ρ_w is the density of water. In highly purified water ($\hbar\omega_{\max} = 3.75 \text{ eV}$ (see, for example, ref. 6 and refs therein)) we obtain $\eta \approx 3 \times 10^{-5}$, whereas in air, at atmospheric pressure, $\eta \approx 4\%$.

Note that equation (13) depends neither on the size R_0 of the nuclearite nor on its velocity provided that equation (11) which may be written

$$\frac{mv^2}{n} \gg (l/R_0)^2 \omega_{\max} \quad (14)$$

is valid. Recalling that the effective radius of a nuclearite is never less than $1 \text{ \AA}$, we find that equation (14) is satisfied in water for any nuclearite whose velocity has not been severely degraded. In air at sea level, equation (14) is satisfied by cosmic nuclearites exceeding a radius of $2 \times 10^{-7} \text{ cm}$ corresponding to a minimum mass of 10^{-5} g . Incidentally, equation (13) does not apply to ordinary meteors that ablate and are consumed before equation (12) is satisfied.

Some recent searches for proton decay can as well be adapted to searching for cosmic nuclearites. For example, the Irvine-Michigan-Brookhaven (IMB) proton decay detector⁵, consisting of 80 kt of purified water surrounded by phototubes at a depth of $1.7 \times 10^5 \text{ g cm}^{-2}$, detects the Cerenkov light produced

by relativistic charged particles as they traverse the detector. The water is highly transparent to visible photons with energies of 2.25–3.75 eV, and the device can detect events involving the production of at least 3×10^4 photons. From Fig. 2, it follows that only nuclearites heavier than 10^{-11} g reach the IMB detector. However, the nuclearite must not only reach the detector, but do so with a velocity greater than 30 km s^{-1} corresponding to a temperature of several thousand K, which means they must be heavier than $\sim 2 \times 10^{-11} \text{ g}$ for detection at IMB. On the other hand, to produce an event rate of $>1 \text{ yr}^{-1}$ within the detector, the nuclearite flux must satisfy $F \geq 3 \times 10^{-5}$, with units (hereafter suppressed) $\text{km}^{-2} \text{ yr}^{-1} (2\pi \text{ sr})^{-1}$. If the IMB detector were programmed to detect slower moving nuclearites ($30\text{--}300 \text{ km s}^{-1}$), the region displayed in Fig. 1 could be explored and perhaps excluded within one year's operation.

Some experiments designed to search for cosmic magnetic monopoles consist of large surfaces of scintillator placed near the Earth's surface. Scintillating material is even more sensitive than water to the passage of nuclearites and can detect nuclearites with masses as small as 10^{-13} g , in the case of an unshielded detector at sea level. The region explorable with a planned detector^{7,8} of area $1,500 \text{ m}^2$ is shown in Fig. 1.

Nuclearites, like meteors, produce visible light as they traverse the atmosphere. Their rate of power dissipation, from equation (2), is $A\rho v^3$. Using the luminous efficiency given by equation (13), we can deduce an altitude-independent expression for their luminosity as a function of their mass

$$L = 1.5 \times 10^{-3} (M/1 \mu\text{g}) \text{ watt} \quad (15)$$

which is valid provided that equation (13) is satisfied. Because of the latter constraint, our analysis does not apply to nuclearites lighter than 10^{-5} g . The visual magnitude of an atmospheric nuclearite at a distance h from an observer is computed from equation (15) to be

$$\mathcal{M} = 10.8 - 1.67 \log_{10} (M/1 \mu\text{g}) + 5 \log_{10} (h/10 \text{ km}). \quad (16)$$

For example, the apparent magnitude of a 20-g nuclearite at a distance of 10 km is $\mathcal{M} = -1.4$, equal to that of the brightest star, Sirius.

Atmospheric nuclearites may easily be distinguished from ordinary meteors. Travelling at galactic velocities ($v \sim 250 \text{ km s}^{-1}$), they are much faster than meteors, which, being bound to the Solar System, move no faster than 72 km s^{-1} relative to the Earth. Moreover, meteors generally emit light only in the upper atmosphere ($\geq 100 \text{ km}$) where they ablate and disintegrate. We show below that the production of light by nuclearites is essentially a low-altitude phenomenon. Thus, the apparent angular velocity of a nuclearite is usually two orders of magnitude greater than that of a meteor.

The constraint imposed by equation (14) can be expressed as an upper limit to the altitude at which nuclearites effectively generate light. Using the fact that the scale height of the atmosphere is $\sim 8 \text{ km}$, we obtain

$$h_{\max} = 2.7 \text{ km} \ln (M/1.2 \times 10^{-5} \text{ g}). \quad (17)$$

Thus, nuclearites with a mass of 10^{-4} g produce their light at altitudes of less than 6 km whereas those with a mass of 10^4 g shine below 60 km. Evidently, the meteoric behaviour of nuclearites is essentially a phenomenon of the lower atmosphere.

From a point on the Earth, the flux corresponding to one annual atmospheric event is given by $F = 2/\pi h_{\max}^2$ at zenith angle smaller than $\pi/4$. (The factor of 2 reflects the difficulty in making daytime observations.) For a large domain of nuclearite masses, this expression which defines the excludable region shown in Fig. 1 is less than the maximum cosmic flux of nuclearites of equation (1). To what extent searches already performed have actually eliminated the excludable region is obscure to us. The largest nuclearite, compatible with the cosmic limit that is likely to be detected from one location during a year of observation, has a mass of $4 \times 10^4 \text{ g}$. The apparent magnitudes of hypothetical cosmic nuclearites range from $\mathcal{M} = +6$ for

nuclearites near 10^{-4} g to $M = -3$ for those with masses near the upper-mass cutoff.

Price *et al.*⁹ recently reported negative results of a search for magnetic monopoles having examined ancient mica for etchable trails of lattice defects produced by cosmic monopoles. Their data provide our only example of an established limit on the cosmic nuclearite flux, arguing that an energy loss, $dE/\rho dx \geq 2.5 \text{ GeV cm}^2 \text{ g}^{-1}$, in the form of nuclear recoil suffices to produce an etchable track. In the case of nuclearites, virtually all of the energy loss is of this kind. With their assumed mean mica burial depth of 5 km, we calculate from equation (9) that an etchable track is produced by any cosmic nuclearite with mass greater than 2.4×10^{-10} g. The mica sample has an area of 13.5 cm^2 and an estimated age of 0.5 Gyr. Thus, the minimal measurable nuclearite flux to which this experiment is sensitive is $F = 1.5$ and its excluded region is shown in Fig. 1.

Etchable tracks in mica are but one example of fossil traces that may be produced by nuclearites in rock. Any nuclearite more massive than 2.4×10^{-10} g can leave an observable track at great depth—an otherwise inexplicable linear astroblem. Neither meteorites, micrometeorites, natural radioactivity nor conventional cosmic radiation can produce such an effect. The tracks produced by nuclearites lighter than ~ 1 g are microscopic and require for their detection sophisticated searches on well-chosen materials in the spirit of the Price *et al.*'s mica experiment⁹. Larger nuclearites can produce visible astroblems. The energy dissipated by a cosmic nuclearite, given by equation (2), is sufficient to melt a cylinder of rock with a radius $R_M = v\beta^{-1/2}$, where $\beta \sim 800 \text{ J g}^{-1}$ is the energy required to heat and fuse 1 g of rock. Thus, $R_M \sim 400 R$. A 1-g nuclearite produces a fossil track with a diameter of $\sim 10^{-2}$ cm. The incidence of such fossils in 10^9 -yr-old rock is $\sim 1 \text{ cm}^{-2}$, if the upper limit on the cosmic nuclearite flux of equation (1) is attained. Still larger nuclearites may be associated with fossils consisting of crushed rock. From the fact that a 1-kt (TNT equivalent) nuclear test in granite produces a spherical crush zone of radius 30 m (ref. 10), we deduce that the radius of the crush zone associated with the passage of a large nuclearite is $\sim 2,000 R$. Thus, a 1-kt nuclearite produces a linear astroblem of diameter 4 m. The maximum possible frequency of such events is 10^{-2} km^{-2} per 10^9 years. We show in Fig. 1 the result of a hypothetical negative hunt for nuclearite fossils, terminating at nuclearite mass of 1.5×10^{18} g or radius 10 cm beyond which the maximum cosmic flux corresponds to less than one collision with the Earth in its lifetime.

The last effect of nuclearite passage that we shall consider is the possibility of nuclearite-induced epiliner earthquakes. Note that the diametric Earth passage of a 1-t nuclearite releases a total energy equivalent to a 50-kt (TNT equivalent) nuclear weapon. (At 30° zenith angle, this is reduced by a factor of 2; at 60° by a factor of 5.) Such nuclearites may collide with the Earth at a rate of no more than 1 yr^{-1} . At teleseismic distances, the seismic signal produced by a point source (weapons test or earthquake) is comparable in magnitude to that produced by a line source of the same total energy release. Indeed, the nuclearite may produce a larger signal, as its seismic efficiency when passing through the dense mantle may be larger than the $\sim 10^{-2}$ efficiency of a well-tamped nuclear shot in granite¹¹. Nuclearite seismic signals are readily distinguishable from other sources: their arrival times, characteristic of a linear sonic antenna, are vastly different from those of a point source. (Remember that the nuclearite traverses the Earth in < 1 min.) Surface waves should be absent, as most of the energy loss takes place in the mantle. Similarly, the production of shear waves is suppressed by a power of c/v , where c is the local sound velocity. Large epiliner earthquakes (body magnitude, $m_b > 5$) can perhaps be 'discovered' in existing seismological data. Thus, the excluded domain shown in Fig. 1 is bounded by $F \geq 10^{-9}$ corresponding to one event in the Earth per decade. The limit on signal size corresponds to an energy deposition equivalent to 1-kg TNT km^{-1} , or a nuclearite mass of 1 kg.

The experiments discussed here, as well as others (such as acoustic detection at sea and the effect of small nuclearites on

airglow) can severely constrain the hypothesis of a cosmic nuclearite flux. In particular, the explanation of the galactic missing-mass problem in terms of nuclearites smaller in size than several centimetres is a hypothesis that is subject to feasible (though highly interdisciplinary) experimental testing.

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1. Farhi, E. *Preprint* CTP 1160, Massachusetts Institute of Technology (1984).
2. Chin, S. & Kerman, A. *Phys. Rev. Lett.* **43**, 1292-1295 (1971).
3. Rubin, V. C. *Science* **220**, 1339-1344 (1983).
4. Opik, E. J. *Physics of Meteor Flight*, 87 (Interscience, New York, 1958).
5. De Rújula, A. *et al. Phys. Rep.* **99**(6), 341-396 (1983).
6. Sulak, L. in *Unification of the Fundamental Particle Interactions*, 619 (Plenum, New York, 1980).
7. Ritson, D. M. *Preprint* UUHEP 82/2, University of Utah (1982).
8. Musset, P. *CERN-EP Internal Rep.* 82-4 (1983).
9. Price, P. B. *et al. Phys. Rev. Lett.* **52**, 1265-1268 (1984).
10. Bolt, B. A. *Nuclear Explosion and Earthquakes*, 190 (Freeman, San Francisco, 1976).
11. Bolt, B. A. *Nuclear Explosion and Earthquakes*, 39 (Freeman, San Francisco, 1976).

A 164-day period in γ -ray bursts from GBS0526-66?

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The γ -ray burst history of GBS0526-66 is unique among observed burst sources¹ being that of the brightest burst on record (5 March 1979) and one of only two from which recurrent bursts (at least 15 of similar temporal and spectral pattern) have been observed (though much less intense² than the first), and the only one whose source direction coincides with that of a previously known object—its 0.1 arc min^2 error box lies within the supernova remnant N49 in the Large Magellanic Cloud³. We have found evidence for a pattern in the recurrence times of these bursts, showing an apparent period of 164 day, which we suggest reflects periodic accretion to a neutron star from an eccentric ($e \sim 0.9$) binary companion. We propose that this system drives two types of bursts: (1) the relatively frequent but weak recurrent bursts produced by either magnetospheric gating, or thermonuclear runaway, of periodically accreting matter around the neutron star, and (2) the much rarer intense bursts, like that of 5 March 1979, produced by tidally-triggered star quakes.

Continued monitoring by the Konus instruments aboard Venera 11 and 12 (from about 14 September 1978 to 27 January 1980) and Venera 13 and 14 (from about 16 November 1981 to 5 April 1983) has revealed² 15 weaker γ -ray bursts, following that of 5 March 1979, with the same celestial position, very similar spectra, and durations ranging from 0.1 to a few seconds. The fluences, S , of these weaker bursts, which varied from $\sim 1.5 \times 10^{-7}$ to $6.5 \times 10^{-6} \text{ erg cm}^{-2}$, were roughly proportional to their duration, and were orders of magnitude less than the $1.3 \times 10^{-3} \text{ erg cm}^{-2}$ observed from the 5 March 1979 burst.

The most striking feature of these recurrent bursts, however, is that their recurrence times, Δ —the time elapsed since the previous burst—show a very high degree of ordering. This is clearly seen in both Table 1 and Fig. 1 (bottom) for the observations of bursts by Venera 13/14 which were of higher sensitivity than those of Venera 11/12, and provided a much more complete sample. Starting from the first burst on 1 December 1981, the recurrence times increased steadily over the next three bursts. Then, on 23 April 1982, the recurrence time abruptly shortened to 0.5 day, and then once again increased over the next six successive bursts. The random probability of nine successive values occurring in one or two sets of any combination of lengths,

ordered with either rising or falling values, is twice the sum of the binomial coefficients, $\sum_{s=1}^8 9!/(9-s)!s!$ divided by the total number of combinations, $9!$, or 0.0028. We must multiply this probability by three, as there are three sets of nine contiguous values in the complete group of 11 values. Thus, we suggest that the chance of this ordering being non-random is 99.2%, or significant at the 2.6σ level.

Moreover, we see from Fig. 1 (bottom) that the values of the recurrence times for the six bursts, beginning with the second burst on 23 April 1982, are roughly proportional to the time of occurrence measured from 23 April 1982. The three previous recurrence times also show an almost linear increase with time. We propose that this ordered pattern of recurrence times may repeat with a regular period. If so, then the observations before 23 April 1982 would suggest that the period is ≥ 140 day, and the later observations would suggest that it is ≤ 350 day. As the two half-day recurrences on 5–6 March 1979 and 23 April 1982 should occur at about the same phase, the period should also be roughly an integral divisor of the 1,145-day interval between them. These constraints limit the possible periods to 143, 164, 191, 229, and 268 day. Folding the data on these periods, we find that the best qualitative fit for all the data is obtained with the period of 164 ± 0.75 day; the best linear relationship between recurrence time and phase is shown with the folded data in Figs 1 and 2 (bottom).

This simple linear relationship agrees qualitatively with all but the event of 31 December 1982, which has a recurrence time about twice that expected. This discrepancy, however, may be owing to a missing burst in October or November 1982, resulting from the fluence being below the detector threshold, or the detector system being temporarily inoperative. The latter possibility would seem likely, if the operating schedule of Venera 13/14 was similar to that of Venera 11/12 on which the operating time of the Konus experiment dropped from almost 90% of the time, during the first half of the mission, to roughly 50%, during the final few months⁴. As the Venera 13/14 threshold appears to be at least a factor of three lower^{2,5} than that of Venera 11/12, the smaller number of recurrent bursts detected in 1978–80 compared with 1981–83—that is, three as opposed to twelve—may result simply from detector threshold differences. For bursts greater than the Venera 11/12 threshold ($> 5 \times 10^{-7}$ erg cm⁻²), there is no evidence for a change in the rate of recurrence, as the same number, were observed during both missions. Thus, both sets of Konus observations could be consistent with regular bursting.

If we also consider the equivalent mean flux (S/Δ) versus time, we see in Figs 1 (top) and 2 (top) that most of the observed emission comes at intervals of roughly 164 day or a multiple thereof. The highest fluxes are strongly peaked close to phase 0.0, and all fluxes greater than twice the minimum of 2.3×10^{-14} erg cm⁻² s⁻¹ occur within a phase interval of 0.33. Moreover, this latter phase interval contains 96% of the time-integrated flux, or fluence, from all bursts observed following that of 5 March 1979.

Assigning a numerical probability to the existence of a 164-day periodicity is very difficult for the following reasons. First, a simple power-spectral analysis of occurrence times would not be expected to yield significant results, because the 16 events were spread over an interval of 1,500 day that included a 1,000-day gap and, thus, only a limited number of long periods could be sampled. If we use the technique of Ferraz-Mello⁶, however, which is designed to accommodate such data gaps, a simple sinusoidal 164-day period is only significant at the 15% level, because the distribution of occurrence times is far from sinusoidal, requiring many higher harmonics, which the limited data cannot resolve. A power-spectral analysis of the equivalent mean flux will also be insensitive to any specific period, as the almost 'delta-function' emission of 0.5-day duration dominates the power spectrum and causes almost equal power to appear at all frequencies.

Our analyses, however, clearly suggest that the highly ordered pattern of recurrence times is non-random at the 2.6σ level and

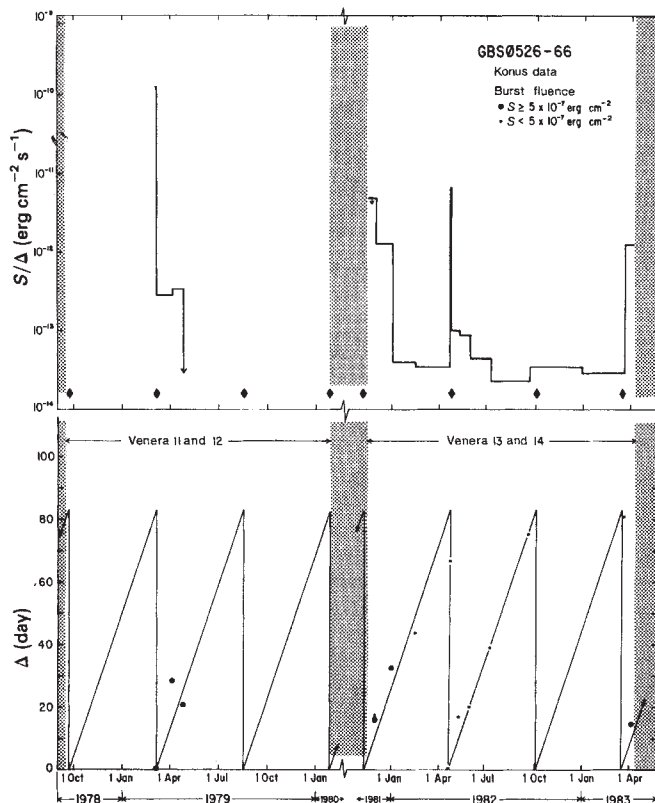


Fig. 1 The distribution of the burst recurrence times, or time between bursts, Δ , shown at the occurrence time of each burst (bottom), and the equivalent mean flux, or the burst fluence divided by the recurrence time, S/Δ , shown for the interval preceding each burst (top). Also shown are diamonds marking 164-day periods and a least-squares linear fit to the recurrence times within that period. All burst fluences greater than the Venera 11/12 threshold of $\sim 5 \times 10^{-7}$ erg cm⁻² are designated by large dots, and those fluences below are shown as small dots.

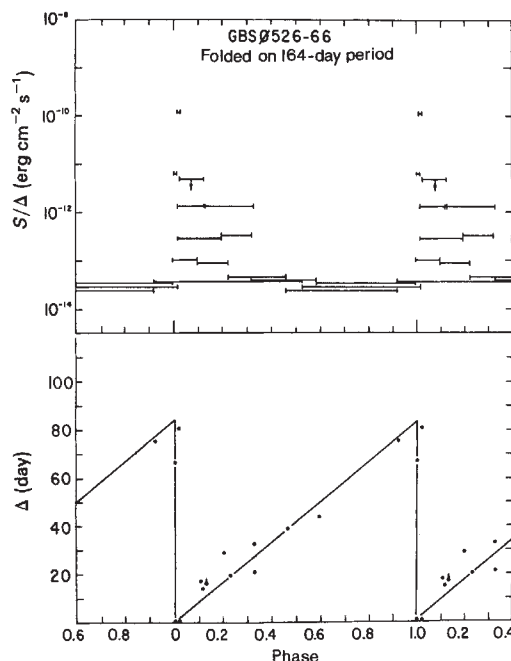


Fig. 2 The phase dependence of the burst recurrence time (bottom), and the equivalent mean flux (top), for a 164-day period. The solid line in the bottom panel represents the best linear fit to the recurrence-time distribution.

Table 1 Bursts from GBS0526–66 observed by Konus²

Burst date (year, month, day)	Julian day (–2443900)	Δ (day)	S (10^{-6} erg cm $^{-2}$)	Phase
79 03 05	38.16	—	1300	0.02
79 03 06	38.76	0.60	6.5	0.02
79 04 04	67.53	28.77	0.7	0.20
79 04 24	88.27	20.74	0.6	0.33
81 12 01	1039.89	>16	6.6	0.13
82 01 02	1072.22	32.33	3.6	0.33
82 02 15	1115.94	43.72	0.15	0.59
82 04 23	1182.66	66.72	0.2	0.00
82 04 23	1183.19	0.53	0.3	0.00
82 05 10	1200.06	16.87	0.15	0.11
82 05 30	1220.03	19.97	0.15	0.23
82 07 08	1258.93	38.90	0.15	0.46
82 09 21	1334.28	75.35	0.15	0.92
82 12 31	1434.68	100.40	0.3	0.54
83 03 21	1515.44	80.76	0.2	0.03
83 04 05	1529.83	14.39	1.6	0.12

Phase based on 164-day period and phase-zero at first burst on 82 04 23.

that this pattern exhibits a 164-day periodicity: burst occurrences are highly asymmetrical—half of them within a very small portion (~ 0.13) of the cycle; $\approx 96\%$ of the recurrent burst energy is emitted within a third of the cycle, and the time between bursts increases almost linearly with phase.

We now consider the possible causes of this pattern of recurrent bursts. GBS0526–66 is generally thought to be a magnetic neutron star^{7,8}. Consistent with this, the cause of the intense burst of 5 March 1979 has been variously attributed to a neutron star quake⁹, thermonuclear runaway of accreted matter on the neutron-star surface¹⁰, or cometary impact¹¹. The three much weaker bursts observed within two months of the 5 March 1979 burst were thought to have been aftershocks or other events resulting from it.

We suggest, however, that the recurrent bursts are separate, although not entirely unrelated, continuing phenomena, occurring both before and after the 5 March 1979 burst. In particular, we propose that this periodic, but asymmetric, pattern of recurrent bursts may result from either thermonuclear runaway or magnetospheric gating of matter periodically accreting around the magnetic neutron star from a binary companion with a 164-day period and a highly eccentric ($e \sim 0.9$) orbit. Both the asymmetric rate of burst occurrence and the increasing recurrence time as a function of phase can be understood if the mass transfer from the companion is essentially limited within a time of the order of a day around periastron. Then the rate of gas accretion to the magnetosphere of the neutron star would be greatest at the time of periastron passage and decrease with time as gas with greater initial angular momentum eventually falls in. With such a decreasing rate of accretion, the individual bursts themselves could result from the filling of some reservoir to a critical mass, which then becomes unstable, producing a burst. The recurrence times between successive bursts will then increase until a new accretion episode is initiated at the next periastron passage 164 day later. The reservoir could be either the accumulation of matter on the surface of the neutron star until it undergoes thermonuclear runaway to produce the γ -ray burst¹⁰, or the accumulation of matter in an accretion disk above the magnetopause, where it remains until an instability develops, dumping it on the surface of the star to produce the burst¹².

The eccentricity of the binary orbit can be estimated from the ratio of the time, p , required for passage through a radian at periastron and the 164-day orbital period, P , by $e \approx 1 - (4\pi p/P)^{2/3}$. If the periastron passage is about 0.5 day—the minimum observed burst separation—then the eccentricity, $e \sim 0.89$, and the size of the Roche lobe would vary by a factor of 20 over the orbital period. Optical searches (F. Cordova, personal communication) show that the GBS0526–66 error box is

empty down to the background brightness of the nebula, giving a limiting apparent magnitude for a companion of ~ 18 m (visual). At the distance (55 kpc) of the coincident supernova remnant in the Large Magellanic Cloud, this limits the mass of a main-sequence companion, for instance, to $\leq 5 M_{\odot}$. We suggest that it is during this brief, but close, approach that the bulk of the matter, accreted over an entire orbital period, flows into the potential well of the neutron star.

We might also expect that if a burst happened to occur on the neutron star close to periastron passage, then the flash heating of the atmosphere of the companion might greatly boost the amount of matter transferred at that time. The efficiency of this process will depend strongly on the orbital phase, as the heating would be proportional to the solid angle subtended by the companion and would vary by roughly three orders of magnitude over the orbital period. The occurrence of bursts as close together as 0.5 day and the tendency for brighter bursts to occur early in the phase may in fact require just such a flash-boosted accretion to provide the very brief but enormous accretion rates needed on those occasions.

The soft X-ray observations place only weak constraints on the accretion rates. Einstein observations of the GBS0526–66 region, both before and after the 5 March 1979 burst, set a 3σ upper limit of $\sim 2 \times 10^{-12}$ erg cm $^{-2}$ s $^{-1}$ on the excess flux of soft (0.5–4.5 keV) X rays observed on 12 April 1979 between the recurrent bursts of 4 and 24 April 1979¹³. This can be compared with the steady flux expected from the accreting matter in magnetospheric gating and thermonuclear runaway models. For a magnetospheric gating process, the expected average radiation flux from matter accreting around the magnetopause at the time of the Einstein observation should be about three orders of magnitude below the soft X-ray limit. But in the thermonuclear model a simple estimate of the bolometric flux at that time suggests that it should be at least $\sim 1 \times 10^{-11}$ erg cm $^{-2}$ s $^{-1}$, which is several times higher than the 3σ upper limit in the 0.5–4.5 keV band. With more detailed calculations, however, the thermonuclear model may still be consistent with the observations.

Finally, we suggest that the accretion of matter from the binary companion may build up stresses within the neutron star that could eventually be released in very powerful quakes, providing energy for much larger but rarer bursts, such as that of 5 March 1979. We may also expect that these bursts would occur preferentially near periastron passage where they could be triggered by the much larger tidal stresses at that time.

In conclusion, we suggest that the pattern of recurrent bursts from GBS0526–66 displays a 164-day periodicity and, if confirmed, this represents the first periodicity seen in γ -ray bursts and the first evidence for a binary companion around a burst source. Further monitoring of this source and study of existing observations are needed to confirm the existence of this periodicity. The strongly asymmetric distribution of burst rates would suggest that there are preferred times for the occurrence of a burst.

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Note added in proof: Since submitting this paper, we have been informed (H. Pedersen and K. Hurley, personal communication) that an optical flash occurring at phase 0.001 by our ephemeris (Table 1) had been detected at the European Southern Observatory–La Silla by their 50-cm telescope (now published¹⁴), seemingly confirming the 164-day period. We predict that periastron passage will occur on 1 January 1985 (± 3 day).

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1. Cline, T. L. *Am. Inst. Phys. Conf. Proc.* **77**, 17–33 (1982).
2. Golenetskii, S. V., Ilyinskii, V. N. & Mazets, E. P. *Nature* **307**, 41–43 (1984).
3. Cline, T. L. *et al. Astrophys. J. Lett.* **255**, L45–L48 (1982).
4. Mazets, E. P. *et al. Astrophys. Space Sci.* **80**, 3–143 (1981).
5. Mazets, E. P., Golenetskii, S. V., Guryan, Yu. A. & Il'inskiy, V. N. *Astrophys. Space Sci.* **84**, 173–189 (1982).
6. Ferraz-Mello, S. *Astr. J.* **86**, 619–624 (1981).

7. Mazets, E. P., Golenetskii, S. V., Il'inskii, V. N., Aptekar', R. L. & Guryan, Yu. A. *Nature* **282**, 587-589 (1979).
8. Ramaty, R., Lingenfelter, R. E. & Bussard, R. W. *Astrophys. Space Sci.* **75**, 193-203 (1981).
9. Ramaty, R. *et al. Nature* **287**, 122-124 (1980).
10. Woosley, S. E. *Am. Inst. Phys. Conf. Proc.* **77**, 273-292 (1982).
11. Colgate, S. & Petschek, A. *Astrophys. J.* **248**, 771-782 (1981).
12. Lamb, F. K. *Am. Inst. Phys. Conf. Proc.* **115**, 179-214 (1984).
13. Helfand, D. J. & Long, K. S. *Nature* **282**, 589-591 (1979).
14. Pedersen, H. *et al. Nature* **312**, 46-48 (1984).

Lightning-induced electron precipitation

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The broadband very low frequency (VLF, 0.3–30 kHz) radiation from lightning propagates in the Earth–ionosphere cavity as impulsive signals (spherics) and in the dispersive plasma regions of the ionosphere and magnetosphere it propagates as tones of descending or rising frequency (whistlers)¹. VLF radio waves propagating in the magnetospheric plasma scatter energetic electrons by whistler-mode wave–particle interactions (cyclotron resonance) into the atmosphere^{2–6}. These electrons, through collisions with the atmospheric constituents, cause localized ionization, conductivity enhancement, visual and ultraviolet light emissions, and bremsstrahlung X rays. We have reported previously on the precipitation of energetic electrons from the radiation belts by the controlled injection from the ground of VLF radio waves^{7,8}. Here we report the first satellite measurements of electron precipitation by lightning. The measured energy deposition of these conspicuous lightning-induced electron precipitation (LEP) bursts ($\sim 10^{-3}$ erg cm⁻²) is sufficient to deplete the Earth's radiation belts and to alter subionospheric radiowave propagation (≤ 1 MHz). A one-to-one correlation is found between ground-based measurements of VLF spherics and whistlers at Palmer, Antarctica, and low-altitude satellite (S81-1) measurements of precipitating energetic electrons.

Detailed measurements of the pulse shape, spectrum, and pitch angle distribution of LEP events have not previously been obtained nor have direct satellite or ground-based measurements of LEP events been obtained within the plasmasphere where most VLF whistler activity occurs⁹. The plasmasphere (average magnetic invariant latitude $\leq 60^\circ$) is characterized by cold plasma densities of 10^2 – 10^4 electrons cm⁻³ and is the region of the magnetosphere which contains the bulk of the radiation belts. The only *in situ* measurement of lightning-induced electron precipitation that we are aware of was reported by Rycroft¹⁰ using rocket data. He observed a single electron burst event having the proper time relationship to an associated whistler and explained the observation as a gyro resonant interaction between ~ 100 keV electrons and a $\frac{1}{2}$ -hop whistler taking place in the magnetospheric equatorial plane. Our *in situ* satellite observations confirm this initial rocket measurement by establishing a one-to-one correlation with a series of strong electron burst events and clarify the details of the precipitating electron temporal behaviour. Electron precipitation due to whistler-triggered emissions outside of the plasmasphere has been observed with balloon-borne X-ray detectors⁶ and ground-based photometers^{11,12}. Indirect evidence of LEP events within the plasmasphere has been derived from amplitude and phase perturbations in VLF signals propagating in the Earth–ionosphere waveguide^{13,14}.

High sensitivity measurements and fine-resolution energy spectra ($2 < E < 1,000$ keV) of the prominent LEP bursts were obtained with a cooled solid-state spectrometer array¹⁵ included as part of the stimulated emissions of energetic particles (SEEP)

experiment on the three-axis stabilized, low-altitude (~ 230 km) S81-1 satellite. The trapped energetic (TE) electron spectrometer ($\pm 20^\circ$ field of view) was aligned perpendicularly to the orbit plane and during these observations was at an angle of 89° to the local magnetic field line. The TE detector had a geometrical factor of 0.17 cm² sr and was cooled (-120° C) to achieve a system noise resolution of 1.2 keV FWHM. An identical detector was positioned to observe electrons with central pitch angles (α) of 52° . The medium energy (ME), precipitating electron spectrometer ($\pm 30^\circ$ field of view) was aligned to the zenith direction and during these observations was at an angle of 25° to the local magnetic field line. The ME geometrical factor was 2.47 cm² sr.

Seven LEP events recorded on 9 September 1982 with the SEEP experiment TE particle spectrometer are shown in Fig. 1 (A–G) with the simultaneous VLF spectra received at Palmer, Antarctica ($L \approx 2.3$). These LEP event signatures are interpreted as follows. The whistler wave in passing through the magnetosphere from north to south alters the pitch angles of energetic trapped electrons which are moving northward and can, therefore, resonate with the VLF wave. This interaction reduces the pitch angles of some of the electrons, lowers their mirror points below the satellite altitude, and produces the first electron pulse of the event. Some of these electrons are then magnetically reflected and some are scattered by the atmosphere, resulting in an electron bunch moving to the Southern Hemisphere where the lower mirroring altitude (due to the South Atlantic anomaly) causes the electrons to encounter the atmosphere. Some electrons are backscattered by the atmosphere and return to the Northern Hemisphere where they are observed as the second pulse in the event. Subsequent reflections and backscattering in the Northern and Southern Hemispheres produce the train of pulses of diminishing intensity which makes up the individual events shown in Figs 1 and 2. These measurements were made 3 days after the strong magnetic storm ($D_{st} = -297$) of 6 September 1982. Magnetic storms of this intensity are known to inject electrons which diffuse into the slot region ($2 < L < 3$) of the radiation belt several days after the storm onset^{16,17}.

In the strong LEP events A, D, and E, electron fluxes are observed to increase rapidly in strength, about 100 times background, in < 200 ms. The envelopes of the individual pulses then decay relatively slowly to background levels over several seconds. Event G is a factor of 10 above background and event F about three times background. Events B and C are relatively weak on the integral energy display of Fig. 1 but are more prominent in the differential energy spectrum ($120 < E < 140$ keV). The reason why the multiflash LEP event E does not show echo pulses may be due to the superposition of four closely spaced LEP bursts. The flux measured with the ME detector ($\alpha \approx 25^\circ$) is a factor of ~ 10 less than the flux measured with the TE detector ($\alpha \approx 89^\circ$) indicating an anisotropic pitch angle distribution peaked near 90° at the satellite altitude. VLF spectra of lightning generated spherics and whistlers were detected in the conjugate hemisphere at Palmer Station, Antarctica, (65° S, 64° W) and are shown in Fig. 1a. The uniform transition in VLF wave intensity at ~ 1.9 kHz indicates a well defined Earth–ionosphere waveguide cutoff frequency. The scaled spherics and whistlers are shown beneath the VLF spectrogram. The dashed portion of a scaled whistler curve is extrapolated based on the observed solid portion and the known properties of more completely defined events such as F. The conjugate of the SEEP satellite (60° S, 98° W) is 34° to the west of Palmer and thus the VLF spectrogram intensity at Palmer is not simply related to the magnetospheric whistler intensity nor to the flux of precipitating electrons.

Whistler-dispersion techniques¹ are used to identify the spherics that precede the delayed whistler signal on the VLF spectrogram. Event E has four similar whistler traces within a 1-s period and is consistent with multiflash lightning. Event C also has two lightning flashes associated with it. An important observation is that the spherics precede the peak of each LEP event by the expected time interval (≈ 0.4 s) for all seven cases.

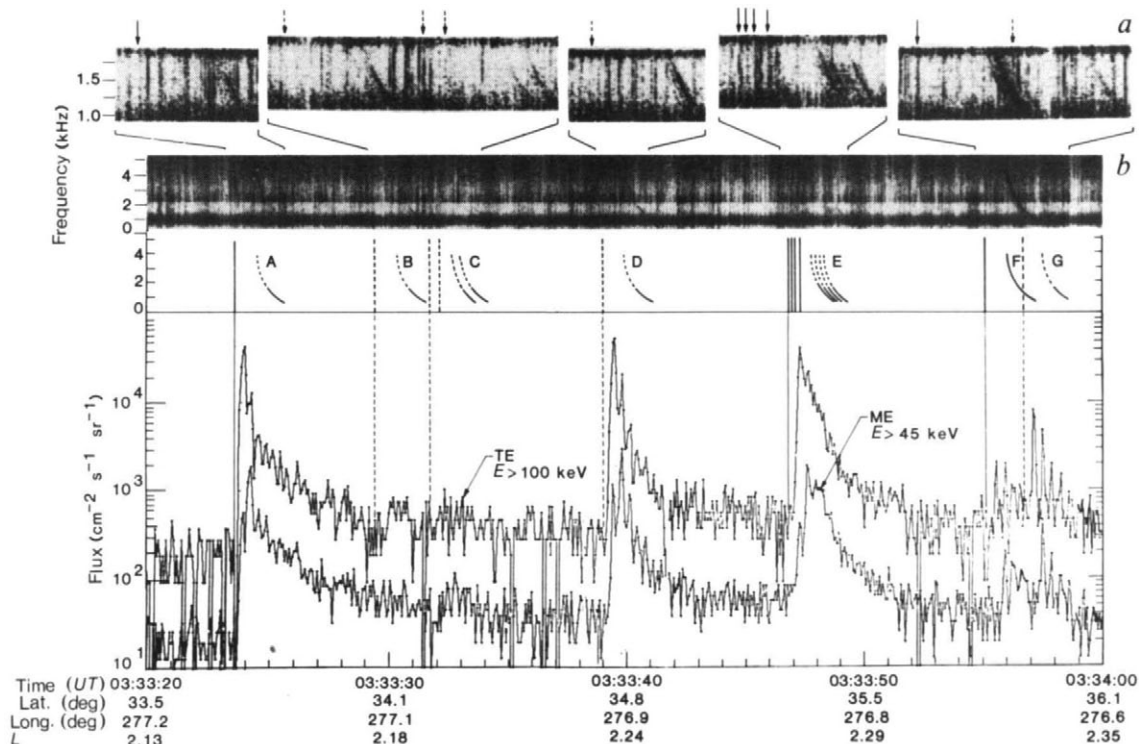


Fig. 1 Energetic electron measurements obtained with the S81-1 satellite are correlated one-to-one with concurrent ground-based VLF whistlers at Palmer Station, Antarctica (65° S, 64° W, $L \approx 2.3$). Panels *a* and *b* show the VLF spectrograms and the scaled whistlers and spherics. The uppermost insets show the Palmer VLF data with an expanded time scale for the seven LEP events. The strong energetic electron bursts labelled A, D and E have peak fluxes that are 100 times background levels. The sampling interval for the energetic particle measurements is 64 ms. The solid lines and arrows represent spherics that were directly identified on these (and other) records, while the dashed lines represent time estimates based on evidence of essentially identical dispersion properties in all events.

Further evidence for the triggering of precipitation by lightning is given by the LEP pulse shapes and spectra. In the pulse shapes of LEP events A, D, F and G, repetitive pulses of constant period and decaying amplitude that follow the LEP peak are conspicuous. Figure 2 shows LEP events F and G on an expanded scale with the associated whistler signals and the subionospherically propagating signals from the transmitter NAA as received at Palmer. The detailed characteristics of the LEP events shown in Fig. 2 include: (1) the rapid rise of electron flux; (2) the subsequent and relatively slow decay of the flux; (3) the in-phase and repetitive pulses on the TE and ME detector (labelled 1-3 for event F and 1-5 for event G); (4) the greater intensity of the near 90° pitch angle flux (TE) compared with the near 0° pitch angle flux (ME); and (5) the weak or completely absent first pulse on the ME spectrometer (indicating a high ratio of drift-loss-cone to direct bounce-loss-cone flux) compared with the subsequent pulses (labelled 2-5 for event G).

A pulse period of 0.32 s is associated with pulses 1-7 of event A. This period agrees with the bounce time of relativistic electrons (~ 150 keV) echoing between conjugate hemispheres at $L = 2.1$. For 175-keV electrons, the relativistic velocity is $0.67c$ and for 125 keV electrons $0.60c$, where c is the velocity of light. As the velocity approaches c the bounce period becomes less sensitive to electron energy variations. For the above energy difference, about five bounce periods (pulses) can occur before the 175-keV electrons are 180° out of phase with the 125-keV electrons. The pulse period of LEP event G at $L = 2.3$ is 0.38 s. This longer period relative to LEP event A agrees with the longer path length of relativistic electrons echoing between conjugate hemispheres at $L = 2.3$ instead of $L = 2.1$.

Examination of the electron energy spectra of LEP events A-G indicates a prominent but broad peak in electron energy between 80 and 200 keV that decreases in energy with increasing L . From comparison of the travel time characteristics of the 9 September 1982, whistlers with similar but more completely defined events recorded on another day at Palmer when whistler-associated precipitation was observed¹⁸, the equatorial electron

density at $L = 2.1$ is estimated to have been $3,200$ electrons cm^{-3} . Assuming ducted propagation the VLF wave frequency would be ~ 4 kHz based on an equatorial gyroresonant interaction with 150 keV electrons. This frequency is in the upper region of the more broadly defined whistlers, such as event F.

Figure 2a shows a small perturbation in the signal received at Palmer from NAA (propagating in the Earth-ionosphere waveguide) which is coincident with the strong whistler and weak LEP burst F. The raw data show the 17.8-kHz signal intensity using a 300-Hz bandwidth filter. Also shown are the smoothed curves of the raw data (dashed lines) outside of the rapid transition interval (± 0.5 s) that were obtained using a 2.4 s averaging filter. The amplitude of the NAA transmitter signal received at Palmer exhibits a fast decrease (< 1.5 s) followed by a slow recovery (~ 10 s). Such a signal perturbation or 'Trimpi' event is associated with lightning-induced electron precipitation which modifies the ionosphere at the ~ 80 km reflection altitude^{13,14}. Considered in isolation, this event is weak because of atmospheric noise at the NAA frequency. However, it is similar in form to a series of stronger events that occurred within several minutes preceding and following the period of satellite data. The strong whistler event F and associated Trimpi event recorded at Palmer are consistent with a relatively strong LEP burst occurring near Palmer. For the other six relatively weak whistler signals no Trimpi events were detected although strong electron precipitation is evident in the SEEP detectors for events A, D, E and G. The simultaneous electron precipitation at the SEEP satellite and near Palmer (Trimpi) which are separated in longitude by 2,000 km, for event F, suggest that a single lightning flash can precipitate electrons at two widely separated locations.

The electron precipitation energy fluxes are estimated to be about 10^2 - 10^5 times the wave energy flux at the equatorial plane, indicating that only a little VLF signal energy is required to perturb the relativistic electrons near the edge of the loss cone enough to cause precipitation³. The energy fluxes reported here are consistent with suggestions that whistlers may cause a

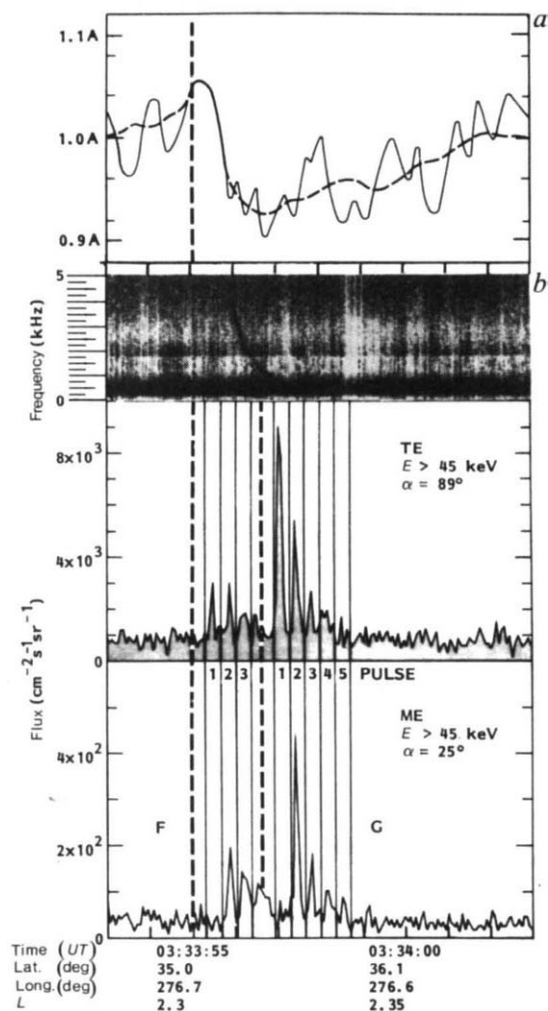


Fig. 2 Expanded view of lightning-induced electron precipitation (LEP) events F and G. The repetitive pulses of constant period labelled 1 to 5 are consistent with the echo period for electrons mirroring between conjugate hemispheres. The vertical dashed line indicates the calculated time of the lightning flash. Panels *a* and *b* show the simultaneous whistler spectrogram and the amplitude of signals received at Palmer from NAA.

significant loss of radiation belt electrons^{3-5,19,20,22}. Assuming that the slot regions of the radiation belts are initially filled after a magnetic storm with an omnidirectional flux of 10^8 electrons $\text{cm}^{-2} \text{s}^{-1}$ for $E > 100$ keV at $L = 2.3$, a single LEP burst (such as event D) can empty $\sim 0.001\%$ of the belt in the region covered by the burst magnetic field lines.

The observations of LEP events provide direct evidence of an important coupling mechanism between terrestrial lightning and relativistic radiation belt electron precipitation. Further study should clarify details of the wave-particle interaction since wide ranges of VLF signal strength and frequencies are present. The global mapping of LEP events can also provide an insight into the penetration of wave energy through the ionosphere and its propagation in the magnetosphere. The role of LEP events in the overall loss rates of trapped electrons is uncertain, because the frequency of occurrence of these events and their relative importance in comparison, for example, with plasmaspheric hiss²¹ is not well known. However, a substantial amount of electrons can be removed in a single event, and additional electron losses can be expected to result from an enhancement of the effective pitch angle diffusion coefficient by whistler waves.

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1. Helliwell, R. A. *Whistlers and Related Ionospheric Phenomena* (Stanford University Press, California 1965).
2. Kennel, C. F. & Petschek, H. E. *J. geophys. Res.* **71**, 1-28 (1966).
3. Inan, U. S., Bell, T. F. & Helliwell, R. A. *J. geophys. Res.* **83**, 3235-3253 (1978).
4. Chang, H. C., Inan, U. S. & Bell, T. F. *J. geophys. Res.* **88**, 7037-7050 (1983).
5. Inan, U. S., Bell, T. F. & Chang, H. C. *J. geophys. Res.* **87**, 6243-6264 (1982).
6. Rosenberg, T. J., Helliwell, R. A. & Katsufakis, K. P. *J. geophys. Res.* **76**, 8445-8452 (1971).
7. Imhof, W. L. *et al. Geophys. Res. Lett.* **10**, 615-618 (1983).
8. Imhof, W. L. *et al. Geophys. Res. Lett.* **10**, 361-364 (1983).
9. Carpenter, D. L. *Radio Sci.* **3**, 719-724 (1968).
10. Rycroft, M. J. *Planet. Space Sci.* **21**, 239-251 (1973).
11. Helliwell, R. A., Mende, S. B., Doolittle, J. H., Armstrong, W. C., Carpenter, W. L. *J. geophys. Res.* **85**, 3376-3386 (1980).
12. Doolittle, J. H. & Carpenter, D. L. *Geophys. Res. Lett.* **10**, 611-614 (1983).
13. Helliwell, R. A., Katsufakis, J. P. & Trimpi, M. L. *J. geophys. Res.* **78**, 4679-4688 (1973).
14. Carpenter, D. L. & La Belle, J. W. *J. geophys. Res.* **87**, 4427-4434 (1982).
15. Voss, H. D. *et al. IEEE Trans. nucl. Sci.* **NS-29**, 164-168 (1982).
16. Lyons, L. R. & Williams, D. J. *J. geophys. Res.* **80**, 3985-3994 (1975).
17. Voss, H. D., Imhof, W. L., Mobilia, J., Gaines, E. E. & Reagan, J. B. *Space Res.* (in the press).
18. Carpenter, D. L., Inan, U. S., Trimpi, M. L., Helliwell, R. A. & Katsufakis, J. P. *J. geophys. Res.* **89** (in the press).
19. Dungey, J. W. *Planet. Space Sci.* **11**, 591-595 (1963).
20. Cornwall, J. M. *J. geophys. Res.* **69**, 1251-1259 (1964).
21. Lyons, L. R. & Thorne, R. M. *J. geophys. Res.* **78**, 2142-2149 (1973).
22. Kelley, M. C. *et al. J. geophys. Res.* (in the press).

A new radiation source for the infrared region

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Spectroscopy in the far-infrared and millimetre-microwave regions has always been hampered by the lack of intense broad-band sources. After several predictions¹⁻⁴ suggesting the possibility of obtaining improved infrared fluxes from an electron source, the Daresbury (SERC) synchrotron storage ring (SRS) has now been shown to produce an intense and very bright source of infrared photons. The infrared port (IR-13(1)) at Daresbury will provide scientists interested in the microscopic structure and dynamics of materials with a radiation source having several unique properties. As well as these properties, we describe here the work that has been done, since January 1984, in characterizing the infrared beam and suggest some wide-ranging potential experimental applications.

Because the photon beam is composed of electromagnetic radiation emitted by a highly relativistic electron beam⁵, the radiation is highly collimated in the forward direction and is strongly linearly-polarized in the orbit plane. Furthermore, it provides a well-defined train of pulses of radiation⁶ of ~ 200 ps in width with a period of either 320 ns (for a single electron bunch) or 2 ns (for 160 electron bunches). The pulse width and shape are entirely independent of frequency, and the inherent photon noise level is expected to be very low compared with that of black-body sources. The beam lifetime is 8-10 h at 2 GeV electron energy. Although the SRS is more intense (in terms of photons emitted per second) than a black-body emitter only in the far-infrared region (below $\sim 50 \text{ cm}^{-1}$), its other advantages—especially its brightness (watts per unit area-solid angle) and its precise time structure—are manifest throughout the infrared.

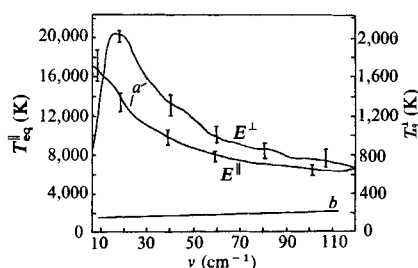


Fig. 1 Equivalent black-body temperatures ($E^{\parallel}$ and $E^{\perp}$) measured for the SRS source (a) (left-hand side, scaled to a 350-mA current) and the mercury lamp (b) (right-hand side) in the region 5–120 cm^{-1} . The effective aperture for both sources was 9 mm.

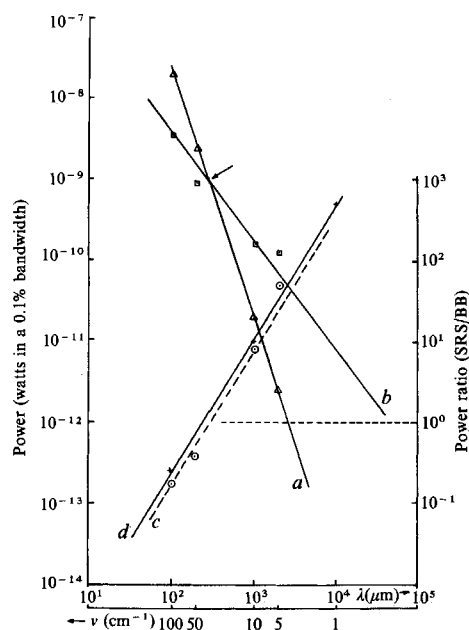


Fig. 2 Comparison of SRS and black-body output powers and power ratios: a, black-body curve at 1,500 K with a 3-mm stop ($A\Omega = 0.045 \text{ cm}^2 \text{ sr}$) (Δ); b, measured SRS power output at 350-mA current and $\pm 20 \text{ mrad}$ vertical collection ($\square$); c, power ratio measured at Daresbury in May 1984 ($\odot$); d, theoretical power ratio⁹ for a total vertical-angle collection and 350 mA ($+$).

Use of a Martin-Puplett interferometer⁷ operating in the 1–140- cm^{-1} (30–4,200 GHz) region in the radiometric mode⁸ has enabled us to measure directly the power and brightness ratios relative to those of a conventional far-infrared source (in this case, a high-pressure mercury arc lamp). Figure 1 shows the brightness measurements ($B(\bar{\nu}) = 2k\bar{\nu}^2 c T_{\text{eq}}$ watts per area-solid angle per bandwidth) made with a source aperture of $\sim 9 \text{ mm}$ —ensuring that the same étendue ($A\Omega$, area-solid angle product) is used in both cases. (The $B(\bar{\nu})$ data have been scaled to the 350-mA current for which our original computed SRS emission^{8,9} was calculated; such currents are easily achieved, although at rather lower electron energies than the standard 1.8 or 2.0 GeV.) The brightness advantage is more than 20:1 at 10 cm^{-1} for a 9-mm aperture. Figure 2 compares the powers ($P(\bar{\nu}) = B(\bar{\nu})A\Omega\Delta\bar{\nu}$) and power ratios with those predicted by Duncan and Williams⁹. For these measurements a mercury lamp aperture of 3 mm was used and $(A\Omega)_{\text{SRS}} \neq (A\Omega)_{\text{Hg}}$. The power ratios are within a factor of five of the theoretical values⁹. The source provides at least an order of magnitude more power at 10 cm^{-1} than a conventional mercury arc lamp, further increasing below 10 cm^{-1} , probably because of coherence effects¹⁰ found when the photon wavelength and the electron bunch length become comparable. Far-infrared lasers¹¹ do, of course, provide output powers in excess of these. The great advantage

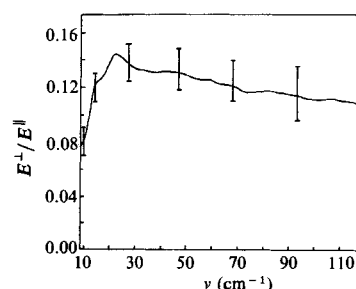


Fig. 3 The integrated polarization ratio $E^{\parallel}/E^{\perp}$ measured for the SRS source in the 8–120- cm^{-1} region (both normalized to a 350-mA current).

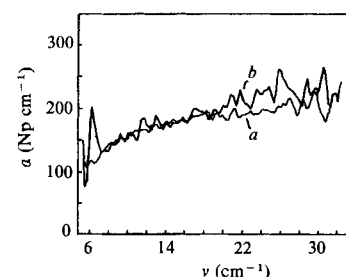


Fig. 4 Comparison of signal-to-noise ratios for a 100- μm sample of liquid water (298 K) in the 4–35- cm^{-1} region, using the SRS source (a), current $\sim 120 \text{ mA}$, and a mercury lamp (b).

of the SRS source is its tunability, in principle from 500 MHz upwards^{1–4}; in practice, we have detected radiation with frequencies down to 30 GHz (1 cm^{-1}) with our present interferometer. No other source provides such continuous radiation throughout the infrared and microwave regions. Also the SRS source has a much smaller pulse width than that of submillimetre lasers, which should give access to faster time-dependent processes^{5,6}, and it is $\sim 86\%$ polarized in the parallel (orbit plane) direction in the 10–100- cm^{-1} region (Fig. 3).

The SRS should provide an ideal infrared source for the following types of experiment:

(1) Experiments requiring enhanced signal-to-noise ratio in the far-infrared/microwave region. For example, we have used the combination of extra photon flux and superior noise characteristics to measure the power absorption coefficient of liquid water (through a pathlength of 100 μm) in the range 4–55 cm^{-1} with signal-to-noise ratio enhancement of ~ 3 (Fig. 4). Such measurements are urgently needed for accurate characterization of water^{12,13} in this important communications region.

(2) Experiments attempting to study the wide range of phenomena occurring in the important spectral gap between the infrared and microwave regions, including the modelling of molecular orientational relaxation processes in polar liquids¹⁴, the study of plasma frequencies in conducting polymers¹⁵, and the study of coherent phenomena^{16,17} and microwave radiation damage¹⁸ in biological systems.

(3) Experiments requiring sub-nanosecond time resolution in the middle and far-infrared regions include those for many important electronic^{19,20}, chemical²¹ and biochemical^{5,6} processes to which the SRS could be applied. One application already proposed by M. Chamberlain (personal communication) is to look directly at the electron kinetics of photothermal ionization in III–V semiconductors using submillimetre (45 cm^{-1}) radiation.

(4) Experiments requiring a photon beam with low étendue ($A\Omega$), either where the sample is small (for example, a single crystal or the small aperture in a high-pressure diamond anvil cell²²) or because the beam must be coupled to a surface whose étendue is also small (as in reflection-absorption infrared spec-

troscopy²³⁻²⁵). Étendues of the order of 10^{-3} cm² sr are obtained for the SRS⁹ at 50 cm⁻¹ and this matches the typical surface experiment²³ quite well. This characteristic of the SRS source should be important, for example, in studies of molecules adsorbed on catalytic (metal) surfaces or in studies of lubricating fluids at high pressures²⁶.

(5) Experiments making use of the beam polarization, which is important, for example, in the study of molecules on surfaces²³⁻²⁵, in the elucidation of optical properties of single crystals²⁷ and in the measurement of birefringence in liquid crystalline materials²⁸.

Besides the intrinsic interest in the study of photon emission from stored electron 'bunches', we anticipate that such novel

experimental features will be relevant in many areas of scientific investigation, having potential applications in the chemistry, physics and biology of materials.

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1. Stevenson, J. R., Ellis, H. & Bartlett, R. *Appl. Opt.* **12**, 2884-2889 (1973).
2. Meyer, P. & Lagarde, P. *J. Phys.* **37**, 1387-1395 (1976).
3. P. Lagarde, P. *Infrared Phys.* **18**, 395-460 (1978).
4. Williams, G. P. *Nucl. Instrum. Meth.* **195**, 383-387 (1982).
5. Munro, I. H. *Synchrotron Radiation and its Application to Chemistry* (Daresbury Laboratory Preprint DL/SRF/P154, 1978).
6. Munro, I. H. *Synchrotron Radiation as a Source to Study Time Dependent Phenomena* (Daresbury Laboratory Preprint DL/SCI/P298E, 1981).
7. Martin, D. H. in *Infrared and Millimeter Waves* Vol. 6 (ed Button, K. J.) Ch. 2 (Academic, New York, 1982).
8. Duncan, W. D. & Yarwood, J. *First Detection of Far-Infrared Photons from a Synchrotron Radiation Source* (Daresbury Laboratory Technical Memorandum DL/SCI/TM32E, 1982).
9. Duncan, W. D. & Williams, G. P. *Appl. Opt.* **22**, 2914-2923 (1983).
10. Michel, F. C. *Phys. Rev. Lett.* **48**, 580-583 (1982).
11. Walzer, K. in *Infrared and Millimeter Waves* Vol. 7 (ed. Button, K. J.) Ch. 3 (Academic, New York, 1983).
12. Afsar, M. N. & Hasted, J. B. *J. opt. Soc. Am.* **67**, 902-904 (1977).

13. Bennouna, H., Cachet, H., Lestrade, J. C. & Birch, J. R. *Chem. Phys.* **62**, 439-445 (1981).
14. Arnold, K. E., Yarwood, J. & Price, A. H. *Molec. Phys.* **48**, 451-469 (1983).
15. Genzel, L., Kremer, F. & Pogtsch, A. *Phys. Rev. B29*, 4595-4601 (1984).
16. Grundler, W. & Keilman, F. Z. *Naturforsch.* **33C**, 15-22 (1979).
17. Fröhlich, H. *Adv. Electron. Electron Phys.* **53**, 85-152 (1980).
18. Grant, E. H. *IEE Proc.* **128A**, 602-606 (1981).
19. Ohyama, T. *J. Phys. Soc. Japan* **51**, 1431-1440 (1982).
20. Pigeon, C. R., Vass, A., Allan, G. R., Prettl, W. & Eaves, L. *Phys. Rev. Lett.* **50**, 1309-1312 (1983).
21. Cureton, C. G. & Goodall, D. M. in *Advances in Infrared and Raman Spectroscopy* Vol. 10 (eds Clark, R. J. H. & Hester, R. E.) Ch. 6 (Wiley, Chichester, 1983).
22. Adams, D. M. & Payne, S. J. *J. appl. Spectrosc.* **27**, 377-382 (1973).
23. Greenler, R. G. *J. chem. Phys.* **44**, 310-315 (1960); **50**, 1963-1968 (1969).
24. Chabal, Y. & Sievers, A. J. *Phys. Rev. B24*, 2921-2934 (1981).
25. Sheppard, N. & Nguyen, T. T. in *Advances in Infrared and Raman Spectroscopy* Vol. 5 (eds Clark, R. J. H. & Hester, R. E.) Ch. 2 (Heyden, London, 1978).
26. Lauer, J. L. *J. Lubrication Technol.* **97**, 145-156 (1975).
27. Sherman, W. F. & Wilkinson, G. R. in *Advances in Infrared and Raman Spectroscopy* Vol. 6 (eds Clark, R. J. H. & Hester, R. E.) Ch. 4 (Heyden, London, 1980).
28. Evans, G. J. *JCS Faraday Trans. II* **79**, 833-845 (1983).

Photocharging of thin films of silver iodide and its relevance to the Daguerre photographic process

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In the Daguerre photographic process¹⁻³, a polished silver-coated plate is exposed to iodine vapour, thereby forming a very thin film of silver iodide, usually about 30-nm thick⁴. After exposure, the image is developed by mercury vapour, which condenses preferentially where light has fallen, amalgamating with silver liberated from the silver iodide (and possibly with the substrate). The remaining silver iodide is dissolved with an aqueous photographic fixing solution (sodium thiosulphate). When viewed at most angles, a positive image of the scene is seen as areas of amalgam (diffusely reflecting), which appear white, on areas of polished silver (specularly reflecting), which appear black. The sharpness with which edges were reproduced in several old daguerreotypes led us to suspect that electrostatic processes might be involved, and we now report two experiments which confirm that.

To demonstrate that mercury vapour in air contains electrically charged droplets, we oxidized a single crystal silicon wafer, 10 cm in diameter, to form a surface film of silicon dioxide about 1-μm thick. A patterned stainless-steel mask (Buckbee-Mears Co., St. Paul, Minnesota) was placed on the oxidized surface, and the open spaces were charged with air ions from a point corona discharge (using a 'Zerostat'⁵ antistatic pistol). Such charge patterns can be rendered visible by immersion in liquid toners, as used in office copying and electrographic printing machines, which consist of electrically charged particles of colloidal carbon of 100-nm diameter suspended in an insulating liquid⁶. Here, the wafer bearing the charge image was placed over a beaker of mercury heated to about 70 °C. When the image was negatively charged, it gradually became visible as droplets of mercury condensed on it (Fig. 1). This experiment clearly demonstrates that positively charged droplets must form in the space between the mercury surface and the wafer, and that they

are attracted to negatively charged areas of the electrostatic image. The fact that some fraction of all finely divided particles and droplets is usually electrically charged is, of course, well known (for example, see Ref. 7).

To demonstrate that those areas of a daguerreotype plate exposed to light do indeed become negatively charged, we prepared some daguerreotype surfaces by sputtering silver on glass plates and exposing them to iodine vapour at room temperature (about 25 °C) for periods of up to an hour in darkness or in red light. The stainless-steel mask now clamped to the surface of the plate was then exposed to direct sunlight for about a second. The exposed plate, after removing the mask, was immersed in liquid toner containing positively charged particles, whereupon black toner particles were immediately deposited uniformly on the exposed parts of the surface. After rinsing in clear carrier fluid and drying, a clear image of black on silver was obtained (Fig. 2). We also prepared scanning electron micrographs to confirm that toner particles were indeed clustered on the exposed areas (Fig. 3). Such liquid-toned images on daguerreotype surfaces could at first be smeared or transferred to adhesive tape. Fixing was accomplished by heating, as the toner also contains a wax for this purpose. The silver amalgam image prepared by the original Daguerre process can also be smeared and transferred easily, and it remains so even after 'fixing' by heating to about 60 °C. These observations suggest that a photographic transfer process could possibly be based on easily prepared Daguerre surfaces.

An alternative explanation is that the surface becomes positively charged during exposure to iodine vapour and that the charge is dissipated in those areas exposed to light. This hypothesis was shown to be invalid, because we found that exposed plates produced no visible image at all when using negatively-charged toners. Furthermore, the deposition of positively-charged toner in the light exposed areas is very uniform. If the area were uncharged, development would occur only around the edges of the uncharged areas⁷. This effect also explains why negatively-charged toner does not give a visible image.

Xerographic layers can typically withstand electric fields due to surface charge of 10-30 V μm⁻¹ (ref. 7); thus we expect the surface potential of a 30-nm thick silver iodide layer to be in the range 300-900 mV. However, we were unable, using a

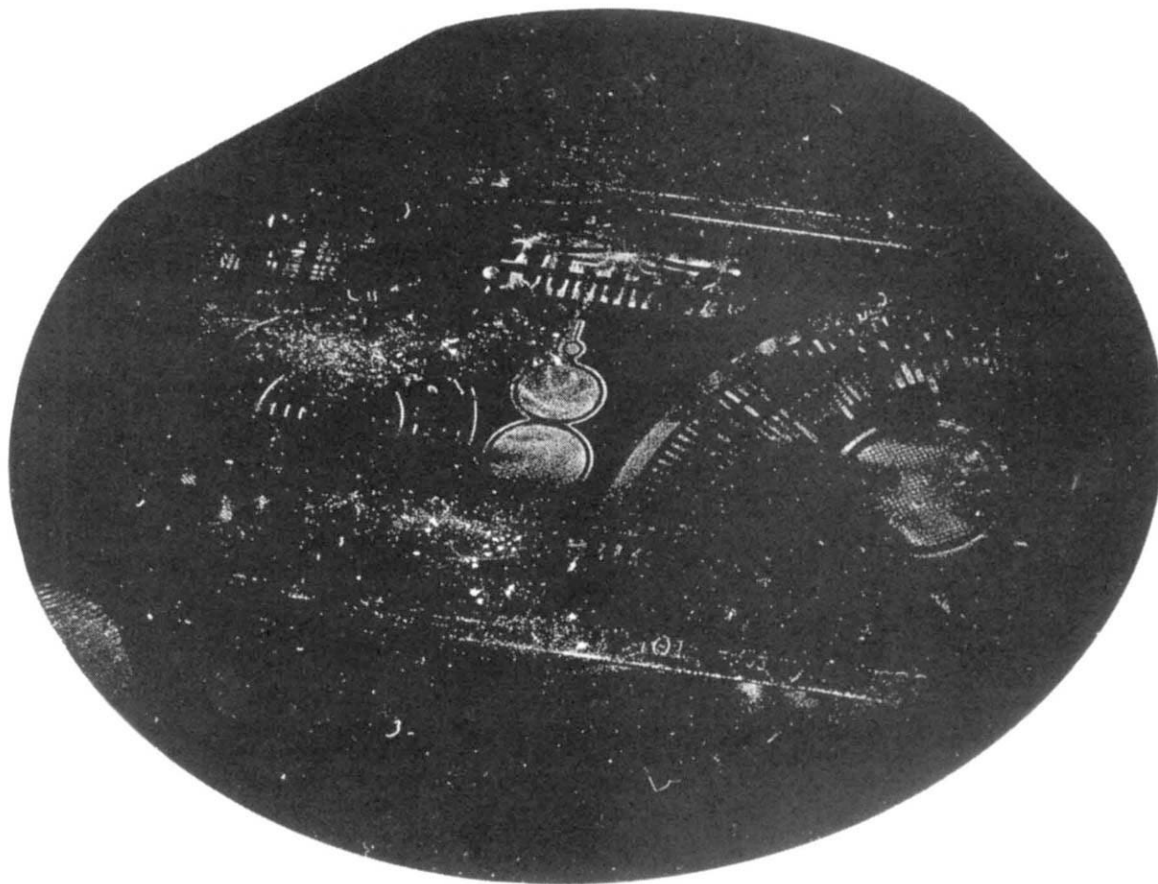


Fig. 1 Image—negatively charged by air ions from a point corona discharge—formed on a surface of oxidized silicon suspended in condensing Hg vapour.



Fig. 2 Image of black toner particles on a surface of iodized silver. The AgI-coated plate, after exposure to direct sunlight, is immersed in liquid toner containing charged particles (Savin Corporation, Stamford, Connecticut).

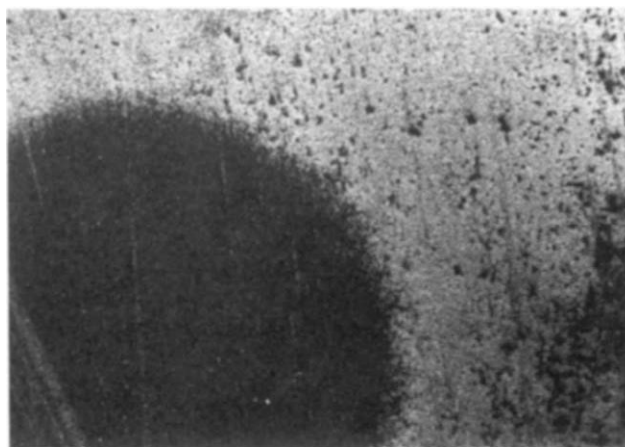


Fig. 3 Scanning electron micrograph of carbon toner on silver iodide.

Monroe electrostatic voltmeter (J. Monroe Electronics Inc., Lyndonville, New York) to detect any effects, due to charge, on the exposed silver iodide films. This was probably because the probe could not be brought any closer than about 100 μm from the surface, and the instrument is not sensitive enough at this distance to detect such low voltages.

It is interesting to speculate about the physical mechanism of the observed optical charging. As is well known, in the normal photographic process with silver halides, exposure to light causes a latent image of metallic silver atoms to be formed and the release of bromine atoms. The bromine atoms are highly unstable, and recombination to silver bromide (fading of the

latent image) occurs rapidly in the absence of a gelatin matrix⁸. In the case of daguerreotype plates, it is possible that the high electron affinity of iodine atoms may be satisfied by diffusion of electrons from the nearby substrate of metallic silver, thus contributing to the negative charge image. Alternatively, photoelectrons ejected from the silver interface may become trapped in the overlying silver iodide layer. Either process may explain the increased sensitivity obtained by adding to the iodine the halogens of greater electron affinity.

Further research may be able to discriminate between these and other possible mechanisms for the photocharging behaviour observed with daguerreotype plates. The use of liquid toners

with positively-charged colloidal particles of white coloration (such as titanium dioxide) will produce daguerreotypes without resorting to the toxic mercury vapour process.

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1. Newall, Beaumont *The Daguerreotype in America*, 115–136 (Dover, New York, 1975).
2. Swan, A., Fiori, C. E. & Heinrich, K. F. J. *Scanning electron Microscopy* 1, 411–424 (1979).
3. Barger, M. S., Messier, R. & White, W. B. *Photogr. Sci. Engng* 26, 285–291 (1982).
4. Pobboravsky, I. *Graphic Arts Res.* 142, 5–13 (Rochester Institute of Technology, Rochester, 1971).
5. US Patent 3,997,817.
6. Dahlquist, J. A. & Brodie, I. J. *Appl. Phys.* 40, 3020–3027 (1969).
7. Schaffert, R. M. *Electrophotography*, revised edn 35, 41–42, 397–406 (Focal, London, 1975).
8. Neblette, C. *Photography, Its Materials and Processes*, 21 (Van Nostrand, New York, 1962).
9. Prohaska, R. & Fisher, A. *Appl. Phys. Lett.* 40(3), 283–285 (1982).

Relaxation of stored mechanical stress along chemical reaction pathways

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Mechanical stress induced in a condensed material relaxes by way of viscous flow, that is by slippage of neighbouring molecules past each other and by the frictional dissipation into heat of the potential energy between them. We show here that this is not necessarily the only way by which energy of deformation can be dissipated. The breaking of chemical bonds within mechanically stressed molecules participating in a chemical reaction provides an alternative and parallel pathway for relaxation and is thus a factor in rheological description which has not been adequately recognized. A chemical change not only alters the composition of the system, and thus the response of its material, but also directly modifies the time scale of the relaxation modes of the molecules participating in the reaction. The involvement of chemical reactions in rheological processes has very broad implications, as most real systems—certainly all living systems—undergo chemical changes, although the rates of these may vary considerably.

Stresses and strains are induced when a force is applied to the surface of a condensed material system. In the resultant deformation, the atoms of which the material is composed are displaced from the potential energy minima characterizing the unstressed state. The internal stresses so generated must balance at each point. Hence, the extent of the displacement of the various atoms depends on the nature and steepness of the local potential energy gradient. For example, a very small displacement will generate the required stress between atoms linked covalently to each other, whereas a much larger shift in position will be needed to generate an equal force between two non-covalently linked first-neighbour atoms. Superimposed on these deterministic, internal, stress-producing interatomic shifts are spontaneous, random, thermal displacements in the material. These are always present, at a finite absolute temperature, even in the stress-free case, and in thermodynamic equilibrium. They involve not only the rapid vibrations of individual atoms about their potential energy minima, but also the slower transitions of assemblies of atoms, that is, of groups, molecules or complexes, from minimum to minimum and back. When stresses generated by externally applied forces are present, they act to bias these transitions and thus to produce permanent deformation or flow. Different materials are distinguished from each other by the number and nature of the groups, molecules or complexes which are capable of substantial change in form or position at the temperature in question within the time scale of interest.

It is often possible to describe a material system as a linear combination of mechanisms for the relaxation of applied stress, with each mechanism corresponding to a degree of freedom of the atoms of which the system is composed, and associated with a time whose magnitude depends on the primary and secondary

structure of the molecules of the system and their relationship to surrounding molecules. Each mechanism makes a contribution of order $k_B T$ to the modulus for instantaneous deformation, where k_B is the Boltzmann constant and T is the absolute temperature. The instantaneous modulus, that is, the stress induced per unit volume by unit strain instantly applied, is thus determined by the number of mechanisms per unit volume. As the number of mechanisms is a measure of the number of degrees of freedom, that is, of the number of atoms per unit volume, the instantaneous moduli of condensed phases are all relatively similar. The differences between material systems arise from the ways in which these mechanisms, essentially constant in total number per unit volume, are distributed over the relaxation-time axis, that is, by the spectrum of mechanical relaxation times.

When deformations are small, or flow is sufficiently slow, all events are linearly additive. It has then been usual to assume that, despite deformation, relaxation and flow, the material remains chemically and rheologically the same at all times. There are neither mechanically-induced chemical changes, nor chemically-generated compositional changes superimposed on the mechanical workings of the system.

The question may, however, be asked, what are the effects on the mechanical relaxation of such a system if its molecules can interconvert chemically¹? These effects, it turns out, transcend the obvious ones resulting from the change in composition². The molecular rearrangements caused by the chemical reaction provide additional pathways along which energy of deformation, stored in the mechanical relaxation mechanisms of the reacting species, can be transferred or dissipated into heat. The instant 'switching on' of a chemical reaction thus has two consequences: (1) the relaxation times characteristic of the mechanical relaxation pathways of the reacting molecules in the absence of chemical reactions are instantly lowered, and (2) the composition of the system and hence the number of mechanisms corresponding to each relaxation time is altered at the rate of the chemical reaction^{1,3,4}.

Therefore, if the change in mechanical response characteristics, during the period of chemical transition, is measured by the dynamic storage and loss moduli, the transients in these parameters should show the effects of the changes, both in the relaxation spectrum and in composition³. Hence, a homogeneous system, already possessing the composition of the reaction in chemical equilibrium and thus not subject to compositional change, will nevertheless produce a transient when the reaction, originally 'frozen', is initiated. In the general case of arbitrary starting composition, of course, the consequences of both effects are experienced.

In order to exemplify these ideas, let us consider a system composed of three rheologically-active macromolecular species which can interconvert. The chemical reactions will be assumed to alter the stiffness of the polymeric chains. For example, let us assume that there is a ring system associated with each monomer, which reduces the flexibility of the chain. Let us further assume that there are catalysts present which can selectively open or close these rings. Let species 1 be the stiffest polymeric species and let each molecule of 1, in dilute solution, contribute a Rouse-like relaxation time spectrum, $\tau_{1(1)} = 1$, $\tau_{1(2)} = 1/4$, $\tau_{1(3)} = 1/9$, ... $\tau_{1(p)} = 1/p^2$... We assume that there are four species of catalysts present: one of these, (21), converts a molecule of 1 into a molecule of 2 by opening the ring structure at every second monomer unit; another, (32), reacts with a molecule of 2 and opens all of the remaining ring structures, converting it into a molecule of 3; another, (23), reverses the action of (32); and the remaining one, (12), reverses the effect of (21). Catalysts (21) and (32), acting simultaneously, take a molecule of 1 directly into one of 3, and catalysts (23) and (12), acting together, cause 3 to revert to 1. Molecules of 3 are thus more flexible than those of 2 and the respective Rouse relaxation time spectra of species 2 and 3 are taken to be $\tau_{2(1)} = 1/9$, $\tau_{2(2)} = 1/36$, $\tau_{2(3)} = 1/81$, ... $\tau_{2(p)} = 1/(3p)^2$, ... and $\tau_{3(1)} = 1/100$, $\tau_{3(2)} = 1/400$, $\tau_{3(3)} = 1/900$, ... $\tau_{3(p)} = 1/(10p)^2$, ... In practice, we shall consider only the first three relaxation mechanisms in

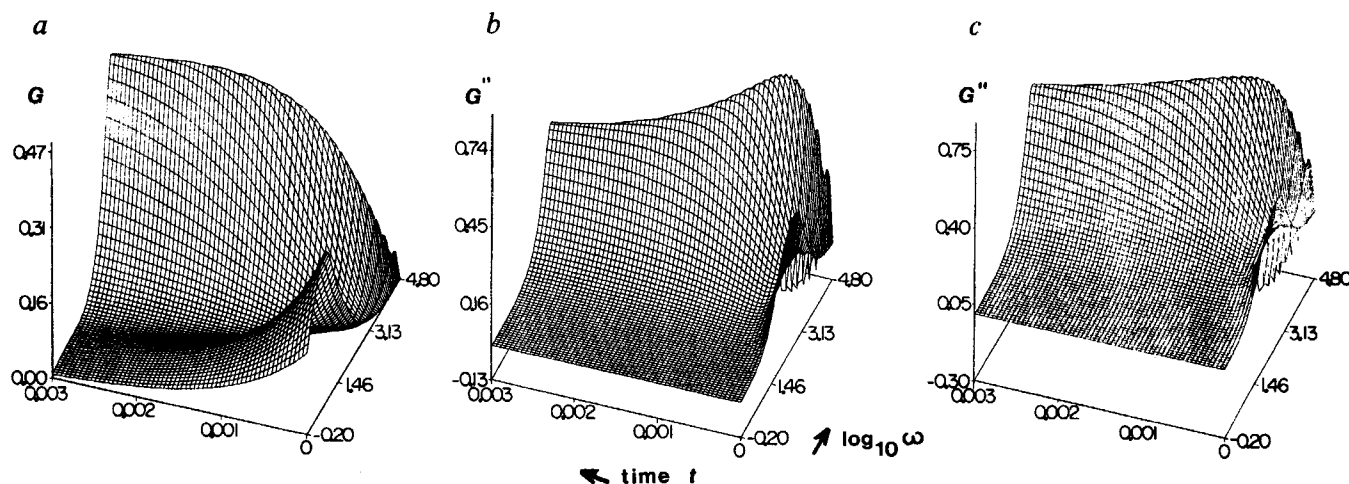


Fig. 1 Frequency (ω) and time (t) dependence of the dynamic shear loss modulus (G'') in the presence of a chemical reaction transient. Units are arbitrary, but the same for *a*, *b* and *c*. The system comprises three macromolecular species, possessing truncated Rouse relaxation-time spectra, undergoing macromolecular interconversions; only the three longest relaxation times are considered in each case. Oscillations started at time $t = -100$. The chemical reaction started at time $t = 0$. The initial composition in *a* matches the composition at chemical equilibrium; in *b* and *c* the initial concentrations of species 1 and 2 are zero. The dissipation modes are: *a*, *b*, no transfer of energy of deformation from reagents to products and total direct dissipation into heat as each stressed molecule reacts; *c*, partial transfer of energy of deformation from reagents to products with the remaining energy dissipated directly into heat. Note that the experimental determination of G'' , at a certain frequency ω , requires a minimum of time of the order $1/\omega$. During this period, the system should remain essentially unchanged chemically. Otherwise, the measurement becomes difficult to interpret. If the mean chemical conversion time is $1/\langle k \rangle$, then the time for measurement, $1/\omega$, must be shorter. Hence only that portion of the theoretical transient in G'' lying at frequencies ω larger than $\langle k \rangle$ can conveniently be verified. In the present example, the chemical transient occurs between $t = 0.0001$ and 0.001 , so that the critical value for $\log_{10} \omega$ lies between 3 and 4.

each case. The time scale is arbitrary and is taken with respect to the longest relaxation time, $\tau_{1(1)}$, which is set equal to one. The three species interconvert according to the following reaction scheme

$$\frac{dn_i}{dt} = \sum_{j=1}^3 k_{ij} n_j; \quad i = 1, 2, 3 \quad (1)$$

where the n_i are the concentrations; the k_{ij} are the rate constants for the conversion of j into i , if $j \neq i$, and k_{ii} is the rate at which i reacts to form the other two species. Thus $k_{ij} \geq 0$ if $j \neq i$, whereas $k_{ii} < 0$. The time t and the k s are on the same reduced time scale as the τ s. The concentrations of the four catalysts described, [12], [23], [32] and [21], stay constant and are included in the appropriate constants k_{ij} . For example, $k_{12} = k'_{12}[12]$, $k_{11} = -k'_{21}[21] - k'_{31}[32][21]$, etc. In our case, let the scheme *K* of rate constants have the following values

$$K = (k_{ij}) = \begin{pmatrix} -1 & 2 & 5 \\ 2/3 & -3 & 5 \\ 1/3 & 1 & -10 \end{pmatrix} \times 10^3 \quad (2)$$

The solution of equations (1) and (2) implies that, at equilibrium, the concentrations n_i^* of the three species are in the following ratio:

$$n_1^* : n_2^* : n_3^* = 15 : 5 : 1 \quad (3)$$

If we now assume that each macromolecule has the same number of mechanisms $\hat{H}$ associated with each of its relaxation times, then the number of mechanisms will be given by $N_{i(p)} = \hat{H} n_i$. Consequently, the number of mechanisms per unit volume, at equilibrium, $N_{i(p)}^*$, will also be in the ratio given by equation (3).

If linear viscoelastic behaviour is assumed and there is no chemical reaction, the stress $\sigma_{i(p)}$ induced in the $N_{i(p)}$ mechanisms by a time rate of strain $d\gamma/dt$ will be given by

$$((d/dt)I + \Lambda)\sigma = k_B TN(d\gamma/dt) \quad (4)$$

where σ and N are vectors having the nine quantities $\sigma_{i(p)}$ and $N_{i(p)}$, respectively, as elements. In equation (4), I is the (9×9) identity matrix, and Λ is a (9×9) diagonal matrix with the nine quantities $1/\tau_{i(p)}$ along the diagonal. The integration of equation (4) is straightforward.

Let the system have a composition $N = N^0$ at the time, $t = 0$, when the reaction (1) is started. With the start of the reaction, N_i in addition to σ and $d\gamma/dt$, becomes a function of time. If this were the only effect of the reaction, the principle for solving equation (4) would not be changed. However, when a molecule of j reacts to form a molecule of i , the energy of deformation stored in the mechanisms $j(s)$ of that molecule must either be transferred to the mechanisms $i(p)$ of the molecule i , which is formed at the rate of the chemical reaction, or be totally or partially dissipated as heat.

To allow for this, we introduce the stored-stress rate of distribution matrix, A . This is a (9×9) matrix whose elements are given by

$$A_{i(p)j(s)} = \alpha_{i(p)j(s)} k_{ij} \quad (5)$$

where $\alpha_{i(p)j(s)}$ is the fraction of the stress stored in a mechanism $j(s)$ which is transferred to the mechanism $i(p)$ of the molecule i as it is formed. Clearly $\sum_p \alpha_{i(p)j(s)} \leq 1$ and $(1 - \sum_p \alpha_{i(p)j(s)})$ represents that fraction of the stress stored in the mechanisms $j(s)$ at time t which is dissipated as heat. At times $t > 0$, equation (4) must be replaced by

$$((d/dt)I + \Lambda - A)\sigma = k_B TN(d\gamma/dt) \quad (6)$$

If we assume that all of the stored energy of deformation is dissipated as heat as the molecules react, the off-diagonal elements of A are all zero. In this case, equation (6) can be written

$$((d/dt)I + \Lambda')\sigma = k_B TN(d\gamma/dt) \quad (7)$$

where Λ' is a diagonal matrix with elements $1/\tau'_{i(p)}$ on the diagonal, given by

$$1/\tau'_{i(p)} = 1/\tau_{i(p)} - k_{ii} \quad (8)$$

As the k_{ii} in equation (8) are negative, $\tau'_{i(p)} \leq \tau_{i(p)}$. The integration of equation (7) is again straightforward.

If γ is taken to be a sinusoidally-varying shear strain of constant amplitude γ_0 and frequency ω , we can compute the real and imaginary components, $G'(\omega)$ and $G''(\omega)$ respectively, of the complex dynamic shear modulus³. Because of the reaction, both $G'(\omega)$ and $G''(\omega)$ become functions of time, at $t \geq 0$, until chemical equilibrium is established. Frequency, ω , is again reduced to the same time scale as τ .

Figure 1 illustrates what happens in the cases discussed above: in Fig. 1a, the initial mechanism concentrations N^0 are equal to those at chemical equilibrium, N^* ; in Fig. 1b and c, the initial mechanism concentrations are $N_{1(p)}^0 = N_{2(p)}^0 = 0$ and $N_{3(p)}^0 = \text{constant}$. Thus, changes in composition when the reaction is switched on occur only in the latter two cases. Each curve in the $t = \text{constant}$ plane gives G'' as a function of the frequency ω ; these curves are zero-order approximations to the relaxation time spectrum, with $1/\omega$ to be regarded as a measure of τ . The curve at $t = 0$ is thus the spectrum characterizing the system at all times before the start of the reaction.

Because, in the case in Fig. 1a, there is no change in N , the transient observed is owing to a switch from the spectrum $\tau_{i(p)}$ to the spectrum $\tau'_{i(p)}$ (equation (7)). The alternative pathway for dissipation of energy of deformation that is suddenly opened through the reaction accounts for the transient in G'' . However, in the case in Fig. 1b, N also changes at times $t \geq 0$. The transient is thus different, but the final states in Fig. 1a, b and c are all the same. Figure 1c illustrates the case of a non-diagonal A , that is, a case where there is also a transfer of energy of deformation, from mechanism to mechanism, as the macromolecules interconvert. The difference between Fig. 1b and c is very small, however, and this seems to be typical of all cases of non-diagonal A s which we have examined.

We have chosen the relaxation times $\tau_{i(p)}$ of each macromolecular species to follow the pattern of a typical Rouse spectrum, but have used in our computations only the first three relaxation modes ($p = 1, 2, 3$) at the long-time end of the spectrum. This accounts for the unusual frequency dependence of G'' , which lacks its usual high-frequency part.

The process by which displacement-induced stresses relax can be viewed as a progressive loss of interatomic correlation. At any instant, a system will be in a definite configuration and all of its component atoms can be identified and assigned definite position coordinates. At that moment there is total correlation, but as time progresses, most of these correlations are lost. Flexible molecules will deform, but correlations between atoms in covalently linked molecules will, on the whole, be maintained. Correlations are lost as a result of two processes: spontaneous thermal agitations and chemical reactions. The effectiveness of thermal agitation depends on the organization of the secondary bonds between the molecules in the system. Systems can range from being almost always perfectly correlated in their secondary bond structure, as in crystals at low temperature, to those suffering an almost immediate loss of identity, as in gases at high temperature. When a chemical reaction is superimposed, some covalent bonds can now break and re-form, as well. As a result, certain additional correlations are no longer maintained and a further pathway for configurational rearrangements, that is, for material displacement and flow, has been gained.

An example will illustrate these ideas. Let AA react with BB to give 2AB. The correlation between the atoms in each of the parts A is maintained, but not those between the two parts of molecule AA, although A does not occur as an independent molecular species. In such a system, relaxation of mechanical stress can take advantage both of the capacity of a grouping A to undergo thermal displacement as part of a molecule of AA, and of the capacity of this grouping to transfer, in a chemical reaction, from AA to another AA or to AB. There is a sense, therefore, in which it is more appropriate to view such a system of molecules AA, BB and AB as a system containing only the entities of maintained correlation, A and B. Mechanical stress relaxes by taking advantage of all mechanisms by which arrangements of entities such as A and B become decorrelated.

We have considered in this analysis only the fate of energy of deformation stored in molecules subject to the 'trauma' of a chemical reaction. During the energetic upheaval involved in such a chemical reaction, we have supposed that all, or part, of the stored mechanical energy is dissipated into heat at the rate of the reaction. We have not considered here the effect that the energy put into the molecules mechanically may have upon the rate of the reaction and its equilibrium, say, by favouring either

the forward or the reverse process (thixotropy or anti-thixotropy)⁵. We have also not considered here the possibility that some of the energy released by the reaction could find its way into mechanical deformation modes and thus produce changes in shape and motion directly, that is, by acting as a converter of chemical energy into mechanical energy via immediate molecular pathways (mechanochemistry⁶), rather than through specialized macroscopic devices such as engines and motors.

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1. Silberberg, A. & Hennenberg, M. *Ann. N.Y. Acad. Sci.* **404**, 159-169 (1983).
2. Stein, R. S. & Tobolsky, A. V. *Text. Res. J.* **18**, 303-310 (1948).
3. Hennenberg, M. & Silberberg, A. *J. Rheol.* **29** (in the press).
4. Hennenberg, M. & Silberberg, A. *J. Rheol.* **29** (in the press).
5. Eliassaf, J., Silberberg, A. & Katchalsky, A. *Nature* **176**, 1119 (1955).
6. Katchalsky, A. *J. Polym. Sci.* **7**, 393-412 (1951).

Ruthenium in the ocean

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Ruthenium, one of the rarest metals of the platinum group, has not previously been investigated in ocean waters and sediments because of its low concentration therein and the lack of sensitive analytical methods. However, the study of ruthenium distribution in the ocean is of interest not only in oceanology but also in geology, geochemistry and cosmochemistry, as it may help in understanding the lithosphere formation processes. We report here the results of ruthenium trace determination in various oceanic environments carried out using a combination of methods including fire assay enrichment and laser photoionization spectroscopy in conjunction with vacuum atomization¹. The ruthenium content in the marine environment fluctuates in the range covering 4-5 orders of magnitude: ~1 p.p.t. (parts per 10¹²) in marine water, tens of p.p.t. in marine biogenic material, 10-100 p.p.t. in bottom sediments and marine phosphorites, and >1,000 p.p.t. in ferromanganese nodules and the Red Sea metalliferous sediments.

As the analytical information on ruthenium is limited, its abundance in the Earth's crust has only roughly been estimated at 10-100 p.p.t. (ref. 2). Using conventional analytical methods for determining the Ru content in natural objects, the best results (detection limits 1-10 p.p.b. (parts per 10⁹))³ have been obtained only after preliminary chemical concentration⁴ or fire assay concentration⁵ of ruthenium from large (100-500 g) samples. The complexity of the multistage chemical treatment of samples and the volatility of ruthenium oxides make such a concentration technique labour-consuming and not very reliable. The method of fire assay concentration of ruthenium in lead alloy is more convenient, and this is what we have used in our experiments.

The fire assay enrichment procedure^{5,6} consisted of fusing 25 g of the sample with litharge (PbO) and appropriate fluxes at 1,000 °C. Ruthenium is collected in a metallic lead alloy (lead button) of mass 30-40 g. After mechanical separation of slags, the lead button produced was reduced to 50-100 mg by oxidizing in a porous vessel (cupel) with an air flow at a temperature of 900-1,000 °C. According to Kolosova⁵ such lead button reduction does not cause ruthenium losses. When analysing seawater, the volume of about 1 l of acidified water was evaporated to dryness, followed by fire assay enrichment of salt. The enrichment factor obtained by this method was 10²-10⁴.

Table 1 Chemical composition and Ru content of investigated samples

Sample	Location [lat., long., depth (m)]	Ru* (p.p.t.)	Fe ₂ O ₃ (%)	MnO (%)	Al ₂ O ₃ (%)	P ₂ O ₅ (%)	Ni (%)	Cu (%)	Co (%)
1. Clayey-siliceous ooze	Northern Pacific [14°23' N, 117°18' W, 4,125]	250	3.25	0.50	11.09	ND	0.019	0.0037	0.0042
2. Clayey-siliceous ooze	Southern Indian Ocean [8°45' S, 102°19' E, 5,300]	150	4.2	1.1	9.5	ND	0.024	0.022	0.0028
3. Metalliferous sediment	East Pacific Rise, [38°56' S, 99°58' W, 4,330]	1,700	26.32	8.30	8.36	ND	0.097	0.12	0.0228
4. Metalliferous sediment	Red Sea, Discovery Deep	7,600	10.5	0.30	0.6	ND	0.001	0.10	0.010
5. Ferromanganese nodule	Northern Pacific, [14°23' N, 117°18' W, 4,125]	3,200	7.93	38.17	4.68	0.21	1.12	0.97	0.15
6. Ferromanganese nodule	Northern Pacific, [10°58' N, 153°22' W, 4,980]	3,500	9.15	37.80	5.05	ND	1.23	1.05	0.24
7. Ferromanganese nodule	Southern Pacific, [22°45' S, 160°50' W, 4,850]	1,600	24.70	22.60	5.95	ND	0.34	0.20	0.40
8. Ferromanganese crust	Mid-Pacific Mountains [20°41' N, 170°32' W, 1,930]	3,800	23.80	29.50	2.33	ND	0.35	0.08	0.65
9. Phosphorite (Pliocene)	Sea of Japan [38°43' N, 130°12' E]	23	2.41	0.04	1.44	27.98	0.002	0.001	0.001
10. Phosphorite (Pliocene)	Mid-Pacific Mountains, [32°16' N, 172°51' E, 370]	170	0.50	0.10	0.20	16.7	0.001	0.001	0.001
11. Phosphorite (Cretaceous)	Mid-Pacific Mountains, [18°31' N, 175°06' W, 1,050]	260	0.38	0.10	0.51	27.9	0.006	0.004	0.001
12. Phosphorite (Holocene)	Namibian shelf, [22°08' S, 13°58' E, 85]	660	0.30	0.001	Traces	31.5	0.0016	0.0003	0.0001
13. Fish bones	Chile shelf, [21°07' S, 70°21' W, 150]	56	1.5	0.02	Traces	29.5	0.0001	0.002	0.0001
14. Whale vertebra	Namibian shelf, [17°24' S, 11°33' E, 120]	90	0.2	0.01	0.25	27.8	0.0003	0.003	0.0001
15. Whale rib	Namibian shelf, [19°24' S, 12°15' E, 130]	350	1.6	0.01	Traces	31.1	0.0005	0.001	0.0001
16. Ocean water	Bay of Biscay, Surface	1.3	70†	40†	1,000†	70,000†	500†	250†	1†
17. Interstitial water of siliceous-clayey ooze	Southern Indian Ocean, [8°45' S, 102°19' E, 5,300]	6.9	—	—	—	—	—	—	—
18. Atmosphere aerosol	Northern Atlantic, [20 March 1983, 5°43' N, 29°13' W- 21 March 1983, 10°37' N, 27°59' W]	910	—	—	—	—	—	—	—

ND, Not determined.

* Ruthenium values are averages of triplicate analyses and analytical precisions (1 σ) are 15 and 25% for solid and water samples respectively.

† After ref. 15 in p.p.t.

The lead produced after cupellation was put into the graphite crucible of the electrothermal atomizer inside a vacuum chamber. After it was evacuated to a pressure of 10^{-6} mm Hg, the crucible was heated to 900 °C. In this case lead evaporated, while ruthenium, because of its extremely low volatility, was concentrated in the residuum. In this way, the matrix was evaporated for 15–40 min. The rest of the sample was atomized with the crucible temperature increasing gradually up to 2,400 °C. The resulting atomic vapour was formed by the cylindrical channel of the crucible into an atomic beam. This beam was irradiated between two plane electrodes by the radiation of three pulsed dye lasers tuned for three-step excitation of ruthenium atoms to the Rydberg state $4d^7 5s(^5F_5) \xrightarrow{392.6 \text{ nm}} 4d^6 5s 5p(^7D_4) \xrightarrow{633.05 \text{ nm}} 4d^7 6s(^5F_5) \xrightarrow{567.33 \text{ nm}} 4d^7 17p$. 20 ns after laser excitation the Rydberg atoms of ruthenium were ionized by an electric field pulse fed to the electrodes. The ions passed through the slit in one of the electrodes and were detected by an electron multiplier. The laser photoionization analytical spectrometer is described in detail in ref. 1.

Figure 1a shows the typical dependence of an ion signal on evaporation time with the atomizer temperature increasing gradually. The content of ruthenium in the sample is determined by integral selective ion signal. To distinguish the Ru selective signal from the total ion signal, the frequency of one laser was tuned off the resonance of the corresponding transition⁷. The ion signal was calibrated using a curve constructed on the basis of copper samples containing a standard quantity of ruthenium. The possibility of such calibration was confirmed by the equality of the signals from copper and lead alloys which had collected ruthenium from standard samples of ruthenium-bearing ore. The detection limit of Ru in a lead bead was about 0.5 p.p.b.

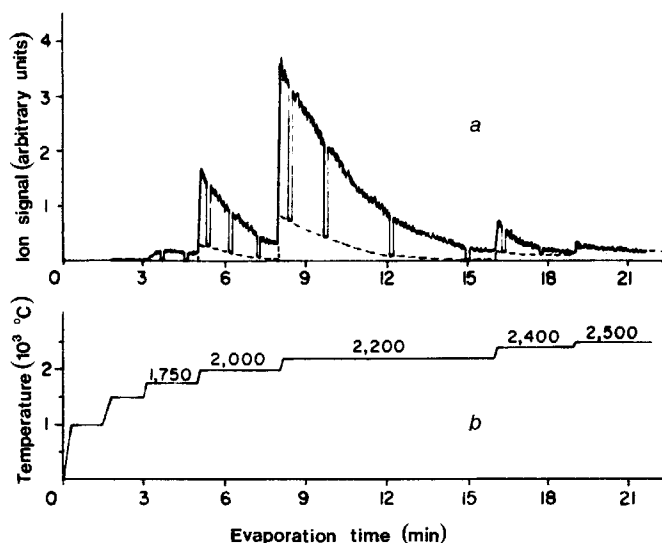


Fig. 1 a, Typical dependence of the ion signal on evaporation time and atomizer temperature for the lead bead of 100-mg weight. The dips are caused by the detuning of the first-step dye laser wavelength from the Ru absorption line to provide a reference baseline. The selective signal of ruthenium is defined by the area between the ion signal curve and the dashed line. b, Time dependence of the atomizer temperature.

Taking into consideration the concentration factor, the Ru detection limits are 1 p.p.t. for solid samples and 0.03 p.p.t. for waters. Typical blanks ranged from less than the detection limit to 7 p.p.t. (solid samples) and 0.2 p.p.t. (waters).

Table 1 lists the investigated materials and the results obtained. The ruthenium content in the biogenic siliceous-clayey sediments from the Pacific and Indian Oceans amounts to 150–250 p.p.t. In the metalliferous sediments enriched in iron and other metals, the ruthenium content is much higher: in the sample from the East Pacific Rise, 1,700 p.p.t.; from the Discovery Deep in the Red Sea, 7,600 p.p.t. Both samples show 7–8-fold enrichment in iron as well as in ruthenium, compared with ordinary sediments. The comparison with other metals listed in Table 1 shows no such correlation.

The metalliferous sediments of the Red Sea are characterized by the high content of several metals, including gold and silver⁸, which could be explained by the corrosive nature of brines percolating rocks and sediments of the rift zone on their way to depressions. Thus, the ore fluids can be enriched with heavy metals in different ratios depending on the local conditions.

Another group of ocean formations enriched in iron, manganese and heavy metals are ferromanganese nodules and crusts⁹. The ruthenium content in investigated nodules varies between 1,600 and 3,500 p.p.t. The lowest content is found in the nodule from the South Pacific. Whereas two samples from the North Pacific show the two-fold enrichment. The maximum of 3,800 p.p.t. is found in the ore crust from a sub-sea mountain of the northern part of the ocean. Comparing these results with the other metal contents shows that the distribution of ruthenium might be correlated with nickel variations in the nodules and cobalt variations in the crust. The high content of ruthenium had been previously determined in standard nodules from Pacific (4,700 p.p.t.) and Atlantic nodules (18,000 p.p.t.)¹⁰. Various kinds of ultramafic rocks contain from 700 to 2,000 p.p.t. of Ru on average¹¹, commensurate with the Ru content in ferromanganese nodules, but lower than that in the metalliferous sediments occurring in the Red Sea.

Phosphate rocks consisting mainly of calcium phosphate belong, too, to the genetically-complex formations on the sea floor¹². In the samples that we studied, ruthenium content varies between 23 and 660 p.p.t. The phosphorite sample from the sub-sea mountain in the Sea of Japan is the lowest in ruthenium containing only 23 p.p.t. In the phosphate rocks from the mid-pacific mountains, the content of ruthenium is much higher (170–260 p.p.t.) and reaches its maximum in the lithified Holocene nodule from the inner shelf of Namibia, 660 p.p.t. The distribution of ruthenium in the analysed phosphorites does not correlate with any of their macro- or microcomponents. The only specific feature of chemical composition of the phosphorite that differentiates it from the other ones is an increased content of organic matter in it¹², which might be the cause of ruthenium concentration.

In the fish and marine mammal bones from the bottom of the shelf regions of the ocean, the ruthenium content ranges from 56 to 350 p.p.t. In the slightly lithified fish bones from the shelf of Chile it reaches 56 p.p.t. and increases in the slightly lithified porous whale vertebrae and rib from the shelf of Namibia to 90 and 350 p.p.t. The behaviour of ruthenium in this case seems to depend on nickel, its content increasing from 0.0001 to 0.0005% respectively.

In the sample of seawater from the Bay of Biscay, ruthenium makes up 1.3 p.p.t. This value is almost the same as the ruthenium content cited in ref. 13 but two orders of magnitude higher than that of palladium determined by Lee¹⁴. Thus, the concentration of ruthenium in seawater is two orders of magnitude lower than in pelagic sediments. Most other heavy metals are characterized by similar feature: their content in seawater is usually 3 or 4 orders lower than in sediments¹⁵.

In the interstitial water of siliceous-clayey ooze from the Indian Ocean, ruthenium content is 6.9 p.p.t. This shows that in the process of pelagic ocean diagenesis, ruthenium is able only to undergo some redistribution between the solid and liquid

phases of sediments. The enrichment of ferromanganese nodules with ruthenium may depend on both its supply from seawater and interstitial water.

Of particular interest is a high content of ruthenium in the atmosphere aerosol sample (910 p.p.t.) taken in the centre of the North Atlantic at the latitude of Sahara. This concentration is about three orders of magnitude higher than in seawater and 4–5 times higher than in pelagic bottom sediments. Such enrichment may be considered as evidence of cosmogenic supply of ruthenium to the ocean owing to high ruthenium content in cosmogenic materials^{16,17}. Similar hypotheses had been suggested for nickel and platinum metals concentrated in the oceanic sediments and in ferromanganese nodules^{18,19}.

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1. Bekov, G. I. & Letokhov, V. S. *Appl. Phys.* **B30**, 161–176 (1983).
2. Reeves, R. D. & Brooks, R. R. *Trace Element Analysis of Geological Materials* (Wiley, New York, 1978).
3. Jäger, H. *Appl. Atom. Spectrosc.* **2**, 1–51 (1978).
4. Khvostova, V. P., Golovnya, S. V. *Zavod. Lab. (Russian)* **48**, 3–7 (1982).
5. Kolosova, L. P. *Zavod. Lab. (Russian)* **48**, 8–12 (1982).
6. Haffty, J., Riley, L. B. & Goss, W. D. *Bull. geol. Surv.* No. 1445 (1977).
7. Bekov, G. I., Yegorov, A. S., Letokhov, V. S. & Radaev, V. N. *Nature* **301**, 410–412 (1983).
8. Hendricks, L. R., Reisbock, R. F., Mahaffey, E. J., Roberts, D. B. & Peterson, M. N. A. in *Hot Brines and Recent Heavy Metal Deposits in the Red Sea* (eds Degens, E. T. & Ross, D. A.) (Springer, New York, 1965).
9. Mero, J. L. *The Mineral Resources of the Sea* (Elsevier, Amsterdam, 1965).
10. Flanagan, F. J. & Gottfried, D. *U.S. geol. Surv. Prof. Pap.* No. 1155, (1980).
11. Yushko-Zakharova, O. E. *Platinum-bearing Ore Deposits* (Russian) (Nedra, Moscow, 1975).
12. Baturin, G. N. *Phosphorites on the Sea Floor* (Elsevier, Amsterdam, 1982).
13. Popov, N. I., Fedorov, K. N. & Orlov, V. M. *Seawater* (Russian) (Nauka, Moscow, 1979).
14. Lee, D. S. *Nature* **305**, 47–48 (1983).
15. Bruland, K. W. in *Chemical Oceanography* Vol. 8, 157–225 (Academic, New York, 1969).
16. Crockett, H. *Geochim. cosmochim. Acta* **36**, 517 (1972).
17. Wedepohl, K. H. *Int. Ser. Monogr. Earth Sci.* **30**, 999–1016 (1968).
18. Pettersson, H. & Rotschi, H. *Geochim. cosmochim. Acta* **2**, 81 (1952).
19. Agiorgitis, G. & Gundlach, H. *Naturwissenschaften* **65**, 534 (1978).

A recently extinct palm from Easter Island

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The former existence of palms on Easter Island has been demonstrated palynologically^{1,2}, but the genus could not be determined from pollen morphology. We now report the discovery on the island of endocarps (shells) from palm fruits which appear to be those of an extinct species related to the Chilean 'wine palm', *Jubaea chilensis* (Molina) Baillon. The endocarps, found in caves, have all been gnawed by rodents, which could have helped to make the species extinct. The radiocarbon age of the endocarps is 820 ± 40 yr BP, which falls within the phase during which deforestation occurred on the island² and the giant statues (*moai*) were erected^{1,3}. Decline of the palm could have contributed to the decline of the *moai* culture.

The pollen record² shows that palms have existed on Easter Island since at least 37,000 BP, but declined to extinction within the past 1,000 years. The fossil pollen (Fig. 1a) is of the monocolpate type with a rough surface, which is common to many palm genera including *Cocos*⁴ and *Pritchardia*⁵, both widespread in the Pacific, and also *Jubaea* which occurs in Chile. The pollen was provisionally assigned to *Pritchardia* (O. Selling, quoted in ref. 1).

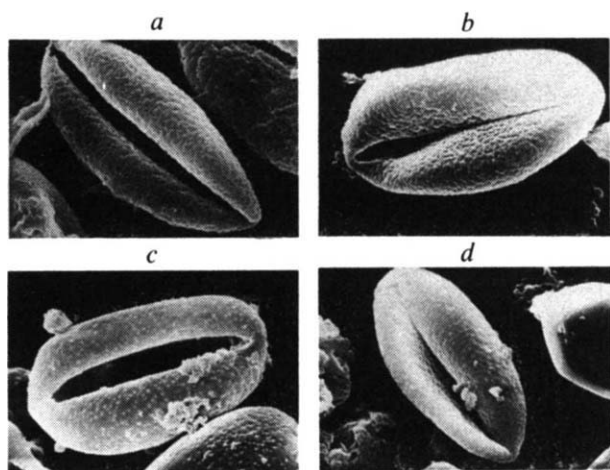


Fig. 1 Pollen grains in the scanning electron microscope. *a*, Fossil pollen from Easter Island; *b*, pollen of *Jubaea chilensis*; *c*, pollen of *Pritchardia minor*; *d*, pollen of *Pritchardia gaudichaudii*.

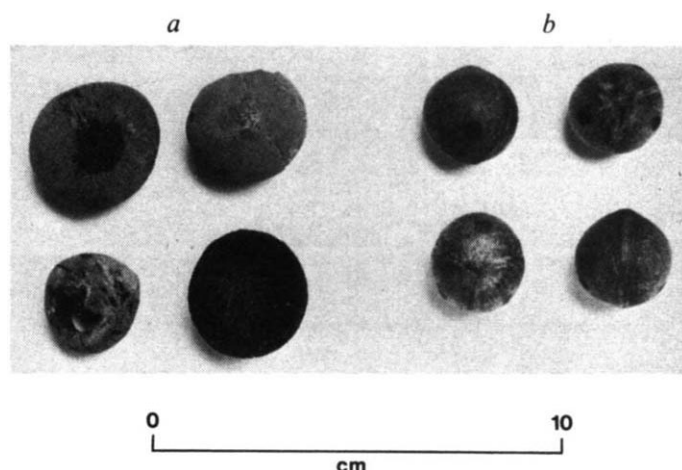


Fig. 2 *a*, Endocarps found in a cave on Easter Island. *b*, Endocarps of *Jubaea chilensis* cultivated in the Temperate House, Royal Botanic Gardens, Kew.

A few *Cocos nucifera* trees exist on the island today but these are of known introduction¹. Palm stumps, believed to be those of coconut, were seen in 1868 (ref. 6), but they could, presumably, have belonged to other species of palm. Legends describe the former existence on the island of a tree called *makoi nau opata* which had an edible fruit, and was used as food by the early settlers, but which no longer survives.

On 20 July 1983, J.-M. Groult and A. Gautier of the *Groupe d'Études et des Recherches Spéléologiques Rouen Ile de Paques* were exploring a newly-discovered cave near Ana Okeke on the cliffs of the Poike peninsula towards the eastern end of the island. On the floor of the cave they found about 30 palm endocarps (Fig. 2*a*), all gnawed. A further single endocarp, similar to the others, was found in August 1983 in a small recess in the face of Ahu Ma'itaki Te Moa on the north coast of the island by M. Symond.

Four of the best-preserved endocarps have been examined. The hard, 2–3-mm thick endocarps are faintly three-angled, three-edged and bear three clearly defined pores. They are clearly endocarps of a palm belonging to the cocosoid major group, and their identity can be narrowed down to the *Cocos* alliance⁷. Of the genera in this alliance, the Easter Island endocarps are closest to those of *Jubaea*, a monotypic genus of Chile, but are, however, consistently different from extant *J. chilensis* (Molina) Baillon (Fig. 2*b*). Three of the four endocarps available for examination are oblate, 30–34 mm in diameter at the equator; the fourth is subspherical, some 25 mm in diameter. The pores lie just below the equator. In *J. chilensis* the pores lie nearer to the base of the endocarp; the equatorial diameter does not exceed 24 mm. We conclude that the Easter Island endocarps belong to a cocosoid palm related to but distinct from *J. chilensis*. Unfortunately generic delimitation in this alliance is based largely on details of inflorescence and flowers; thus it is not possible to indicate whether the Easter Island palm is an extinct species of *Jubaea* or whether it represents a distinct genus.

We performed ¹⁴C dating on several of the fruits. Although the samples appeared to be well preserved, the soluble organic fractions, which may have represented postmortem contamination by foreign organic material, were removed by a series of alternating digestions in hot (80 °C) acid (2 M HCl) and alkali (0.5 M KOH). This decontamination procedure was followed by washing to neutral pH with distilled water. The residual carbon recorded a conventional radiocarbon age value (SRR-2430) of 820 ± 40 yr BP. Conversion of this radiometric age estimate via dendrochronological calibration⁸ places a 95% (2σ) confidence on the fruits having grown at some time in the calendar span AD 1040 to 1280.

The ratio of stable carbon isotopes (¹³C/¹²C) in plant tissues can provide in many instances a clear indication of the metabolic characteristics of the species^{9,10}. Unfortunately in this respect the recorded enrichment value (δ ¹³C_{PDB} = −21.2‰) falls firmly in the overlap between those ranges of ¹³C enrichment that can be recognized as indicative of the C₃ (Calvin cycle), C₄ ('dicarboxylic acid' cycle) or CAM (crassulacean acid metabolism) photosynthetic pathways.

The toothmarks on the endocarps are consistent with gnawing by rodents. The main rodent on the island today is the Norwegian rat, *Rattus norvegicus*, thought to have been introduced since Europeans discovered the island in AD 1722 (refs 1, 11). The Norwegian rat is believed to have ousted an earlier colony of Polynesian rats. In archaeological excavations at Anakena on the north-east coast of the island, we recovered bones of the Polynesian rat *Rattus concolor*, probably introduced by the early settlers. It is probable that this species gnawed the endocarps found in the cave, although the toothmarks are rather small to be those of rats (A. J. Stewart, personal communication). The same excavations produced numerous fragments of palm endocarps similar to those under discussion, suggesting that the fruits were also part of the human diet.

The pollen of *J. chilensis* (Fig. 1*b*) is similar to the fossil pollen (Fig. 1*a*) found in swamps on the island. Both pollen grains have a rugulate surface. The fossil pollen differs, however, from that of the two species of *Pritchardia* with which it has been compared (Fig. 1*c, d*). Both *Pritchardia* species have pollen grains with a fossulate, not rugulate surface.

From the pollen record², it appears that the extinct palm was important in the island vegetation; it may have been the dominant tree in the lowlands. Deforestation occurred in the past 1,000 yr (ref. 2); most of the giant statues were erected during the same period^{1,3}. Man is believed to have reached the island about AD 400¹, so it is possible that the extinction of the Easter Island palm was caused by a combination of direct deforestation and prevention of reproduction, the latter resulting from eating of the fruits by man and the introduced rats.

Not all members of the *Cocos* alliance have large erect stems, but if the extinct palm was similar in morphology to *J. chilensis*, it would have made suitable levers for the movement of the *moai*; its smooth cylindrical trunk could have been cut into rollers for the same purpose. The construction of *moai* ceased suddenly in AD 1680 (ref. 1); this may have been caused by the extinction of the palm.

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- Heyerdahl, T. & Ferdon, E. N. Jr (eds) *Reports of the Norwegian Archaeological Expedition to Easter Island and the East Pacific* Vol. 1 (Forum, Stockholm, 1961).
- Flenley, J. R. & King, S. M. *Nature* **307**, 47-50 (1984).
- Ayres, W. S. *J. polynes. Soc.* **80**, 497-504 (1971).
- Thanikaimon, G. *Institut Français de Pondichery. Travaux de la Section Scientifique et Technique* **11**, 1-286 (1970).
- Selling, O. *Bernice P. Bishop Museum, Hawaii, Spec. Publ.* **38** (1947).
- Palmer, J. L. *J. R. geogr. Soc.* **40**, 167-181 (1870).
- Moore, H. E. *Gentes Herbarum* **11**, 27-140 (1973).
- Stuiver, M. *Radiocarbon* **24**, 1-26 (1982).
- Smith, B. N. & Epstein, S. *Pl. Physiol.* **47**, 380-384 (1971).
- Bender, M. M., Rouhani, L., Vines, H. M. & Black, C. C. *Pl. Physiol.* **52**, 427-430 (1973).
- Metraux, A. *Bernice P. Bishop Museum, Hawaii, Bull.* **160** (1971).

Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*

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The *per* locus of *Drosophila melanogaster* has a fundamental role in the construction or maintenance of a biological clock. Three classes of *per* mutations have been identified: *per*¹ mutants have circadian behavioural rhythms with a 29-h rather than a 24-h period, *per*^s mutants have short-period rhythms of 19 h, and *per*⁰ mutants have no detectable circadian rhythms¹⁻⁴. Each of these mutations has a corresponding influence on the 55-s periodicity of male courtship song⁵. Long- and short-period circadian rhythm phenotypes can also be obtained by altering the dosage of the wild-type gene⁴: for example, females carrying only one dose of this X-linked gene have circadian rhythms with periodicities about 1 h longer than those carrying two doses. In a previous report⁶, cloned DNA was used to localize several chromosomal rearrangement breakpoints that alter *per* locus function. The rearrangements all affected a 7-kilobase (kb) interval that encodes a 4.5-kb poly(A)⁺ RNA. We report here that when a 7.1-kb fragment from a *per*⁺ fly, including the sequences encoding the 4.5-kb transcript, is introduced into the genome of a *per*⁰ (arrhythmic) fly by P element-mediated transformation, circadian rhythmicity of behaviour such as eclosion and locomotor activity is restored. The transforming DNA complements *per* locus deletions and is transcribed, forming a single 4.5-kb poly(A)⁺ RNA comparable to that produced by wild-type flies.

The 7.1-kb *Hind*III fragment contained in phage ZW106 (represented by a wavy line in Fig. 1) was inserted into Carnegie 20 (ref. 7) to form plasmid pCPI (Fig. 1). A mixture of p π 25.1 (ref. 8), which contains a functional P-factor, and pCPI, which contains a defective P-factor that can be rescued by p π 25.1 and a wild-type *rosy* gene (*ry*⁺) that affects eye colour, was microinjected into the region of pole cell formation of preblastoderm *y per*⁰; *ry*⁴² embryos. Survivors were pair-mated with *y per*⁰; *ry*⁴² flies, and F₁ progeny were scored for wild-type eye colour, which indicates successful germ-line integration of pCPI sequences (see Fig. 1 legend). Transformed lines were maintained as heterozygotes, with respect to the transforming DNA, by back-crossing to *y per*⁰; *ry*⁴² flies.

One such transformed line, strain *per*⁰; P1.48C (*y per*⁰/Y; P1.48C/+; *ry*⁴²/*ry*⁴² males, and *y per*⁰/*y per*⁰; P1.48C/+; *ry*⁴²/*ry*⁴² females), carries a single transposon insertion at polytene chromosomal position 48C (chromosome 2R) by *in situ* hybridization. Hybridization of pCPI sequences to restriction digests of total genomic DNA from flies carrying P1.48C indicates that the transforming DNA is present in a single copy, and has the structure predicted from the restriction maps in Fig. 1 (data not shown). Derivatives of this strain of flies were subjected to a series of behavioural tests to determine whether

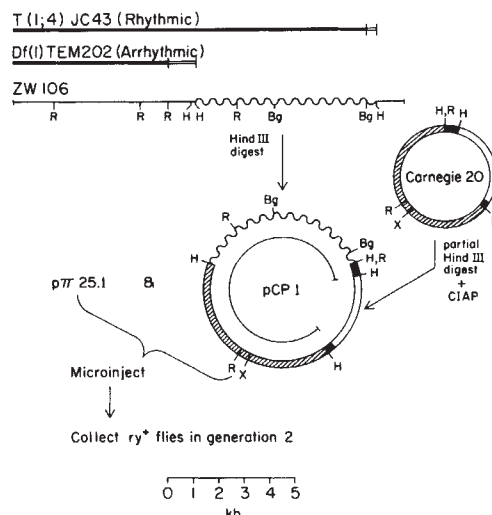


Fig. 1 Physical map of *per* region DNA and the construction of transforming DNA, pCPI. The positions of two chromosomal rearrangements, *T(1;4)JC43* and *Df(1)TEM202*, are shown relative to phage ZW106 (ref. 6). Solid bars represent DNA still present and in the wild-type configuration, while the location of each chromosomal rearrangement breakpoint is shown as an open bar⁶ (see text for details).

Methods: Transforming DNA was constructed as follows: ZW106 was digested to completion with the restriction endonuclease *Hind*III (H, *Hind*III; R, *Eco*RI; Bg, *Bgl*II; X, *Xho*I). The 7.1-kb fragment (shown as a wavy line in ZW106) was purified from a low-melting point agarose gel. Carnegie 20 (ref. 7) was partially digested with *Hind*III and treated with calf intestinal alkaline phosphatase (CIAP) to prevent self-religation. The 7.1-kb fragment was ligated to the partially digested vector and introduced into *Escherichia coli* (HB101). Transforming DNA sequences were mapped with restriction endonucleases and one, pCPI (shown in figure), was chosen for microinjection into *Drosophila*. pCPI contains the recombinogenic ends of a truncated P-factor⁷ (■), *per* locus DNA (—), a wild-type *rosy* gene (⊞), and a combination of DNA sequences from *E. coli* and the *Drosophila melanogaster* white locus⁷ (□). The part of pCPI that is inserted into the *Drosophila* genome by P-factor-mediated transposition includes both *per*⁺ and *rosy*⁺ DNA, and is represented by I-I. pCPI was mixed with p π 25.1, which mobilizes the P-factor associated with pCPI in *trans*⁸, at a 5:1 concentration ratio (350 μ g ml⁻¹ final concentration), and the mixture was injected into *Drosophila* embryos of the genotype *y per*⁰; *ry*⁴². Germ-line integration of pCPI DNA sequences results in a heritable change in eye colour (from *rosy* to wild-type). Because both *per*⁺ and *rosy*⁺ DNA sequences are integrated as a unit, flies with *ry*⁺ eye colour were subsequently tested for expression of behavioural rhythms. (For further details, see text.)

transformation of rhythmic behaviour accompanied transformation of eye colour.

Figure 2 shows eclosion profiles for three strains of flies. Two aspects of circadian behaviour were tested: first, the ability of a population of flies to entrain to a light-dark cycle (12 h of light followed by 12 h of dark); and second, the ability of populations of flies to free-run, or continue to behave rhythmically and synchronously, in the absence of the entraining light-dark cycle. In the experiments of Fig. 2, flies were kept in constant darkness from day 6 until the end of the experiment.

Figure 2a shows that flies carrying one dose of a wild-type (Oregon-R) *per* locus (*y per*⁰/+; *ry*⁴²/+ females) entrain to a light-dark cycle and continue to show circadian eclosion rhythms in constant darkness. The period of these rhythms is ~24 h during entrainment, and 25 h in constant darkness (see Fig. 2 legend). As expected¹⁻³, *per*⁰ flies (*y per*⁰/Y; *ry*⁴²/*ry*⁴² males, and *y per*⁰/*y per*⁰; *ry*⁴²/*ry*⁴² females) eclose arrhythmically in both sets of conditions (Fig. 2b). Flies in the population designated *per*⁰; P1.48C (Fig. 2c) differ from those in the *per*⁰ population only by the presence of the transforming DNA on one of their second chromosomes, at 48C (*y per*⁰/Y; P1.48C/+;

Fig. 2 Temporal profiles of eclosion (emergence of adults from pupal cases) for populations of: *a*, wild-type females ($y\ per^0/+; ry^{42}/+$); *b*, per^0 males and females ($y\ per^0; ry^{42}/ry^{42}$); and *c*, $per^0; P1.48C$ males and females ($y\ per^0; P1.48C/+; ry^{42}/ry^{42}$). Eclosion was monitored simultaneously in the three different populations by manually collecting (at 2-h intervals) the adults that had emerged in culture bottles maintained at 25 °C. Developing cultures were entrained to a cycle of 12 h light/12 h dark for 3 days before the start of collections. This light-dark (LD) cycle was continued for the first 5 days of collections, then cultures were maintained in constant darkness (DD) for the remainder of the experiment. The LD to DD transition is indicated by the arrow above record *a*. Collections during DD were made under a low-intensity safelight (a 15 W incandescent light with a Kodak GBX-2 filter) which does not affect *Drosophila* circadian rhythms^{1,2}. During entrainment to LD 12:12, 24-h period lengths were estimated for the rhythms of wild-type (*a*) and $per^0; P1.48C$ (*c*) populations by measuring the time elapsed between medians of successive peaks of eclosion. By the same procedure, wild-type and $per^0; P1.48C$ populations have free-running period lengths (in DD) of 25 ± 1.1 h and 26.5 ± 1.9 h, respectively. Similar estimates (24–25 h for wild-type and 27–28 h for $per^0; P1.48C$) were obtained from a spectral analysis of the data (Univariate Spectral Analysis BMDP P1T, University of California, Los Angeles; see ref. 11 for methods). Spectral analysis revealed no periodicity in the eclosion profile for per^0 flies.

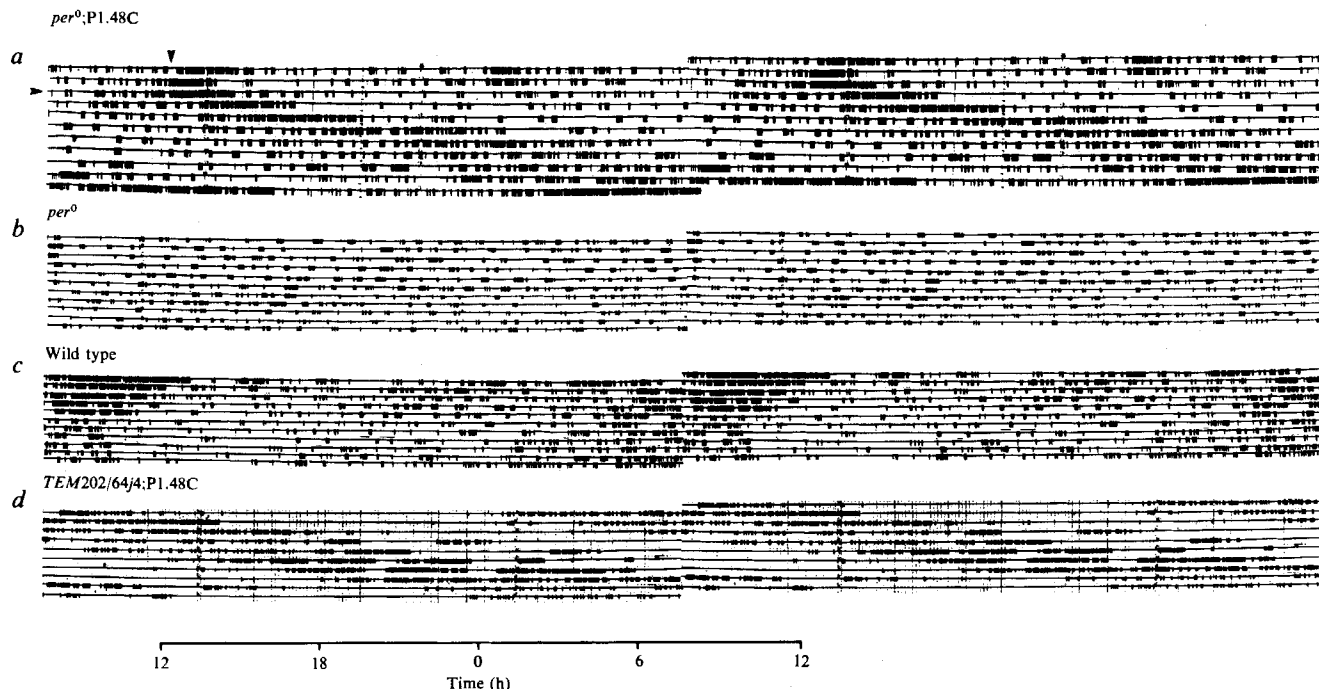
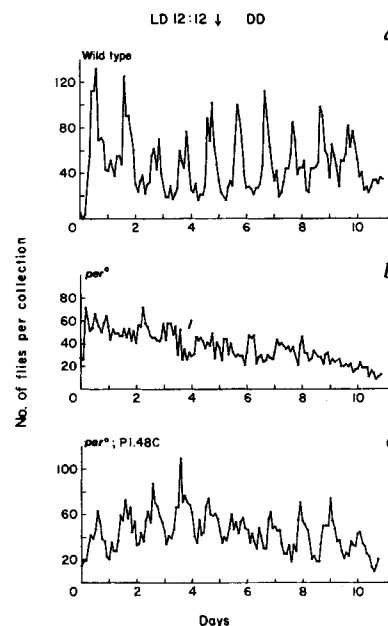


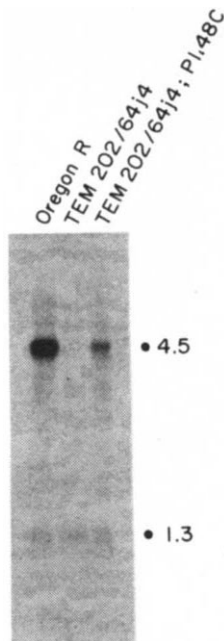
Fig. 3 Records of locomotor activity from individual flies: *a*, $y\ per^0/Y; P1.48C/+; ry^{42}/ry^{42}$ male; *b*, $y\ per^0/y\ per^0; ry^{42}/ry^{42}$ female; *c*, Oregon-R wild-type male; and *d*, $Df(1)TEM202/Df(1)64j4; P1.48C/+$ female. Data were collected by maintaining individuals, at 25 °C, in cylindrical plastic tubes (containing a small amount of medium) positioned between a light-emitting diode and a phototransistor of the monitoring device¹². The diode used emits light in a range (peak emission 900 nm) that does not affect circadian rhythms of locomotor activity¹². When a fly's movement disturbed the light beam, an all-or-none signal was generated which, when amplified, triggered a pen deflection on an Esterline Angus event recorder. Records were constructed by plotting successive days (24 h) of activity one below the other, then plotting twice the resulting chart of activity¹³. Thus, each record is shown twice, but the second record in every pair is displaced upwards 1 day on the right so that a continuous 48-h interval is shown on each horizontal line. In *a*, the first 3 days of the record show entrainment of the $per^0; P1.48C$ fly to a cycle of 12 h light/12 h dark (LD 12:12). Lights were switched on at 0 and off at 12 h. The final 'lights off' signal of the LD cycle is indicated by the intersection of the two arrows (12.00 on day 3). Thereafter, the fly remained in continuous darkness. For *b*, *c* and *d*, only activity in constant darkness is plotted. Period length was determined by measuring the time elapsed between offsets (cessations) of daily bouts of activity¹. All flies were entrained to LD 12:12, with 'lights on' and 'lights off' at the times indicated above, for at least 2 days before each activity recording began.

ry^{42}/ry^{42} males, and $y\ per^0/y\ per^0; P1.48C/+; ry^{42}/ry^{42}$ females). In sharp contrast to per^0 flies, $per^0; P1.48C$ flies eclose rhythmically during entrainment and continue to eclose rhythmically in constant darkness (Fig. 2c). The period of eclosion rhythms during entrainment is 24 h, whereas the period of free-running rhythms is ~27 h (see Fig. 2 legend).

Representative locomotor activity records for individual $per^0; P1.48C$, per^0 and Oregon-R flies are shown in Fig. 3; each

horizontal line of each record represents 48 h. Individual $per^0; P1.48C$ adults entrain to a light-dark cycle and continue to behave rhythmically in constant darkness (Fig. 3a). The first three days of the activity record in Fig. 3a ($y\ per^0/Y; P1.48C/+; ry^{42}/ry^{42}$ male) illustrate entrainment to a cycle of 12 h of light followed by 12 h of dark. Bursts of locomotor activity occur at dawn and dusk. The light-dark cycle ends on day 3 at 12.00. Bouts of activity in constant darkness continue to occur rhythmically.

Fig. 4 Transcriptional activity of P1.48C. Poly(A)⁺ RNAs from flies of the genotypes shown (5 µg per lane) were electrophoresed through 1% agarose/2.2 M formaldehyde gels and transferred to Biodyne A membrane (Pall Ultrafine Filtration). The 7.1-kb *Hind*III fragment from phage ZW106 (Fig. 1) was cloned into the Riboprobe vector pSP64 (Promega Biotec). The autoradiogram shows results from the hybridization of a single-stranded, ³²P-labelled, 1.5-kb RNA probe. The probe was initiated at the SP6 promoter of the vector and terminates at a *Bst*EII site within the 7.1-kb *Hind*III fragment; this *Bst*EII site is located 1 kb to the left of the rightmost *Bgl*II site shown in the ZW106 map of Fig. 1. This probe labels two RNAs after washing at 65 °C in 5 mM NaCl, 2 mM NaPO₄ pH 7, 0.1 mM EDTA, 0.1% SDS. The 1.3-kb RNA (indicated) is present in all samples, including flies deficient for 10 kb of DNA lying between the breakpoints of *Df(1)TEM202* and *Df(1)64j4*. As these flies lack the 7.1-kb *Hind*III fragment, the 1.3-kb RNA cannot be transcribed from this DNA⁶. The 4.5-kb RNA is absent from these flies, but is present in wild-type flies (Oregon-R), and is found in reduced amounts in flies of the genotype *Df(1)TEM202/Df(1)64j4*; P1.46C/+. A densitometric analysis of shorter exposures of the autoradiograph indicates a 10-fold difference in the level of the 4.5-kb RNA between *Df(1)TEM202/Df(1)64j4*; P1.48C/+ and wild-type flies; a fivefold reduction from wild type would be expected in flies carrying two copies of P1.48C. Because the hybridization probe in these experiments is single-stranded, the direction of the transcription of the 4.5-kb RNA is from left to right along the 7.1-kb *Hind*III fragment shown in Fig. 1. Hybridizations using labelled RNA probes synthesized from the 7.1-kb *Hind*III fragment cloned in the opposite orientation fail to detect any homologous RNA species in the three genotypes shown.



transforming DNA produces this RNA, two lines of *Df(1)TEM202/Df(1)64j4* aneuploid flies were constructed, one carrying the transposon insertion (that is, *Df(1)TEM202/Df(1)64j4*; P1.48C/+). Figure 4 shows that the 4.5-kb RNA is expressed in aneuploid flies only if P1.48C is present; it also demonstrates that the 4.5-kb RNA is much more abundant in wild-type than in *Df(1)TEM202/Df(1)64j4*; P1.48C flies (see Fig. 4 legend also).

Our analysis of *Drosophila* strain *per*⁰;P1.48C proves that a 7.1-kb *Hind*III fragment derived from a wild-type fly contains a functional *per* locus, and behavioural analysis of *Df(1)TEM202/Df(1)64j4*;P1.48C flies shows that the transforming DNA can complement *per* locus deletions. *Df(1)TEM202/Df(1)64j4*;P1.48C flies are still completely deficient for two transcription units neighbouring the 7.1-kb *Hind*III fragment; these map near the *T(1;4)JC43* chromosomal rearrangement breakpoint and code for a 1.1- and a 3.2-kb poly(A)⁺ RNA⁶. Because *Df(1)TEM202/Df(1)64j4*;P1.48C flies are rhythmic, it can be concluded that neither transcript has an essential role in the production of circadian behavioural rhythms.

The 7.1-kb *Hind*III fragment was chosen for these transformation experiments because it appeared to contain a single transcription unit that forms a 4.5-kb RNA⁶. As the 4.5-kb poly(A)⁺ RNA is in fact produced by the P1.48C transposon insertion, this RNA appears to be required for expression of behavioural rhythms.

As judged by an analysis of eclosion and locomotor activity rhythms, P1.48C restores the ability of *per*⁰ flies to entrain to light-dark cycles and to express rhythmic behaviour in constant darkness. The only measurable difference between the rhythms of wild-type and *per*⁰;P1.48C flies is the length of the free-running period. The finding that P1.48C transformants have long-period free-running rhythms is not particularly surprising given that period length is known to be sensitive to *per* locus dosage, with a 50% reduction in wild-type gene dosage increasing free-running rhythmic period length by 1 h. Because the level of expression of transforming DNA can be strongly influenced by chromosomal location in *Drosophila*^{9,10}, we suspect that P1.48C produces less than wild-type levels of a functional *per* gene product. Consistent with this interpretation, we find that *Df(1)TEM202/Df(1)64j4*;P1.48C/+ flies produce 10 times less than the wild-type level of 4.5-kb RNA. An analysis of the relationship of rhythmic period length and abundance of the 4.5-kb RNA among several strains of flies independently transformed with the 7.1-kb *Hind*III fragment is under way.

We thank Julie Pan for technical assistance, Laurel Eckhardt, Simon Kidd and Norton D. Zinder for criticism of the manuscript, Joseph Grossfield for the loan of a micromanipulator for DNA injection experiments, and John Lis for instruction in microinjection techniques. This work was supported by grants from the Andre and Bella Meyer Foundation and the NIH to M.W.Y.

Note added in proof: A second *Drosophila* strain independently transformed with the 7.1-kb *Hind*III fragment, produces rhythms with close to wild-type periodicity. Of 15 such flies tested, 14 produced locomotor activity rhythms with a period 1 h shorter than flies transformed by P1.48C. A molecular analysis of *per* (ref. 6) has also been initiated by Reddy *et al.*¹⁴.

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1. Konopka, R. & Benzer, S. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2112-2116 (1971).
2. Young, M. W. & Judd, B. H. *Genetics* **88**, 723-742 (1978).
3. Smith, R. F. & Konopka, R. J. *Molec. gen. Genet.* **183**, 243-251 (1981).
4. Smith, R. F. & Konopka, R. J. *Molec. gen. Genet.* **185**, 30-36 (1982).
5. Kyriacou, C. P. & Hall, J. C. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6729-6733 (1980).
6. Bargiello, T. A. & Young, M. W. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2142-2146 (1984).
7. Rubin, G. M. & Spradling, A. C. *Nucleic Acids Res.* **11**, 6341-6351 (1983).
8. Spradling, A. C. & Rubin, G. M. *Science* **218**, 341-347 (1982).
9. Spradling, A. C. & Rubin, G. M. *Cell* **34**, 47-57 (1983).
10. Goldberg, D. A., Posakony, J. W. & Maniatis, T. *Cell* **34**, 59-73 (1983).
11. Enright, J. T. in *Handbook of Behavioral Neurobiology* (ed. Aschoff, J.) 21-39 (Plenum, New York, 1981).
12. Jackson, F. R. *J. Neurogenet.* **1**, 3-15 (1983).
13. Pittendrigh, C. S. *Cold Spring Harb. Symp. quant. Biol.* **25**, 159-184 (1960).
14. Reddy, P. *et al. Cell* **38**, 701-710 (1984).

cally with a period of ~27 h; each bout of activity occurs ~3 h later than that of the preceding day. Comparable results were obtained with 7 of 8 additional *per*⁰;P1.48C transformants, each monitored for a minimum of five free-running daily cycles (mean period = 28.1 ± 1.2 h). Thus, both eclosion rhythms and locomotor activity rhythms have approximately the same period.

Figure 3b shows the locomotor activity of a *per*⁰ fly (*y per*⁰/*y per*⁰; *ry*⁴²/*ry*⁴² female) in constant darkness. As with the eclosion behaviour of *per*⁰ populations, no rhythmic activity can be seen. Figure 3c shows a representative locomotor activity record of a free-running Oregon-R fly (male); the period of the rhythm in constant darkness is slightly less than 24 h. The period of the rhythms of such euploid, Oregon-R males is about an hour shorter than that of females carrying a single dose of *per*⁺ (Fig. 2); this result is expected because *per* is X-linked and dosage-compensated⁴, and period length is sensitive to *per* locus dosage⁴.

Df(1)TEM202/Df(1)64j4 females are arrhythmic and homozygously deficient for a 10-kb interval that includes the 7.1-kb *Hind*III fragment⁶. To determine whether the transforming DNA sequence in P1.48C can genetically complement this *per* locus deletion, a new group of flies (*Df(1)TEM202/Df(1)64j4*; P1.48C/+ females) was constructed. Figure 3d shows a representative locomotor activity record for a free-running *Df(1)TEM202/Df(1)64j4*;P1.48C fly; the fly is rhythmic, the period of the rhythm in constant darkness being 27 h. Of 25 flies tested, 24 produced comparable rhythms, with a mean period of 27.6 ± 2.4 h. No measurable difference could be found between the rhythms of *per*⁰;P1.48C and *Df(1)TEM202/Df(1)64j4*;P1.48C flies.

A 4.5-kb poly(A)⁺ RNA is transcribed from the 7.1-kb *Hind*III fragment in wild-type flies⁶. To determine whether the P1.48C

Influence of the method of drug administration on analgesic response

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The appropriate control group in studies of placebo-induced analgesia has not been established. A traditional control has been a 'no treatment' or natural-history group. In some studies, the natural-history group receives a hidden infusion of vehicle, a physiologically inactive substance such as saline solution, to eliminate differences in expectation of the outcome on the part of the experimenter^{1,2}. To evaluate whether 'hidden' as well as open infusion of vehicle can elicit a placebo response, we have now tested a different natural-history group, one which received an infusion of vehicle from a syringe pump controlled by a programmable timer. A comparison of these two control groups provides evidence that hidden infusion of vehicle can elicit a placebo response. Use of this new control group also permitted a clear distinction between a naloxone-antagonizable component of placebo analgesia and naloxone antagonism of endorphin-mediated analgesia induced by surgical stress. Our study underscores the power of the placebo and emphasizes that even the most subtle cues can elicit a placebo response.

Ninety-six patients underwent standardized surgery for the removal of impacted third molars. (The surgical procedure has been described in detail elsewhere^{3,4}.) The duration of the experiment, measured from the onset of local anaesthesia, was 5 h. Each patient was randomly assigned to receive, after surgery, a double-blind injection of one substance—naloxone, naloxone vehicle or morphine—via an indwelling intravenous line. Naloxone (10 mg) or naloxone vehicle were each administered to three groups of 12 patients. Each group received the randomly assigned substance by one of three methods: (1) by a person at the patient's bedside (open infusion, patient aware that a substance was being administered); (2) by a person in an adjacent room ('hidden' infusion^{1,2}); or (3) by the preprogrammed infusion pump (machine infusion). Morphine, included to assess the relative potency of placebo, was administered by machine infusion to two groups of 12 patients, one group receiving 8 mg and the other 12 mg of the drug. The average time of substance administration, 3 h after onset of anaesthesia, was the same for all groups. The effect of each intervention was determined as the change in pain 50 min after administration with respect to the pain intensity 10 min before administration. For the present study we have defined the natural-history group as those patients who received vehicle by machine infusion, placebo as the open administration of vehicle, and placebo analgesia as a decrease in pain, in comparison with the natural history group, produced by a placebo.

Mean pain intensity decreased similarly in the two groups receiving either open or hidden infusion of vehicle, but increased after machine infusion of vehicle (Fig. 1). Mean pain also increased after administration of naloxone, regardless of the route. Finally, pain decreased after machine infusion of both doses of morphine.

To demonstrate a naloxone-antagonizable component of placebo-induced analgesia, a 2×2 [(substance: naloxone, vehicle) $\times$ (route of administration: machine, open)] analysis of variance was performed. There was a significant interaction between substance and route of administration ($F(1, 44) = 8.07$, $P < 0.01$). Planned orthogonal comparisons of group means⁵ showed that pain was significantly decreased after open vehicle (placebo) ($F(1, 44) = 15.87$, $P < 0.001$) in comparison with the machine-infused vehicle (natural history) and naloxone groups

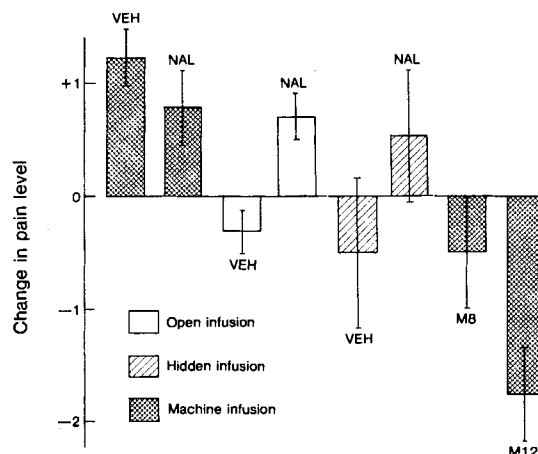


Fig. 1 Changes in pain intensity (mean $\pm$ s.e.) 50 min after the administration of morphine (M), vehicle (VEH) or naloxone (NAL), as compared with pretreatment baseline. Pain was measured with a 10-cm visual-analogue scale^{10,11} every 20 min after surgery. The position of the mark, in millimetres, provided the pain measure. The three methods of administration were open, 'hidden' and machine infusion. Pretreatment baseline pain intensities were: naloxone, open 4.9, hidden 5.8 and machine 6.1; vehicle, open 6.5, hidden 6.0 and machine 6.2; and morphine (machine only) 8 mg (M8) 6.2, and 12 mg (M12) 5.2. There were 12 patients in each group.

(Fig. 1). The significant decrease in pain in the open-vehicle group, in comparison with machine-infused vehicle, demonstrates that a placebo response was elicited. Pain intensity in the three groups that received naloxone and the group that received machine-infused vehicle did not differ significantly ($F(1, 44) = 0.85$, not significant; NS), and route of administration had no effect on the naloxone groups ($F(1, 44) = 0.010$, NS). In other words, pain was significantly increased after the administration of naloxone in comparison with open administration of vehicle, but not in comparison with machine infusion of vehicle.

Open infusion of naloxone produced an increase in pain whereas open infusion of vehicle produced a decrease, suggesting that there is a naloxone-antagonizable component of placebo-induced analgesia. That machine infusion of naloxone had no hyperalgesic effect relative to machine infusion of vehicle demonstrates that the higher pain intensity after open naloxone, in comparison with open vehicle, is due to naloxone antagonism of placebo-induced analgesia and is not an effect of naloxone on the natural history of post-operative pain. Our previous report that naloxone has a hyperalgesic effect³ may be explained also by the fact that we did not include an appropriate natural-history group.

Pain level in the hidden-vehicle group was also significantly less than in the group receiving vehicle by machine infusion ($F(1, 44) = 6.55$, $P < 0.02$). It did not differ significantly from the pain level in the openly-infused vehicle (placebo) group ($F(1, 44) = 0.03$, NS). These data suggest that hidden infusion of vehicle may be accompanied by unintentional cues that elicit a placebo response, whereas machine infusion of vehicle does not provide such cues. Surprisingly, in post-experiment interviews, patients were unable to identify cues signalling when hidden infusions had occurred or what substance had been infused. Thus, subtle cues, of which a patient may not be consciously aware, can significantly influence therapeutic outcome.

The magnitude of the analgesia produced by 8 mg machine-infused morphine was indistinguishable from that produced by placebo ($F(1, 88) = 0.024$, NS). Machine infusion of 12 mg morphine produced a decrease in pain, however, significantly greater than that produced by placebo ($F(1, 88) = 4.08$, $P < 0.05$)⁶. These observations together suggest that the potency of our placebo (open vehicle) is equivalent to approximately 8 mg

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morphine. This equivalent is larger than we reported in an experiment in which hidden infusion of morphine was used⁷. The difference between that experiment and the present one can presumably be explained by a summation of morphine and placebo effects introduced during hidden infusions.

In conclusion, the present study demonstrates that placebo-induced analgesia for post-operative pain is potent, and can be antagonized by naloxone, a relatively specific opiate antagonist⁸. This finding confirms our earlier study⁹, in which we did not have a natural-history group. Patients were categorized, based on their response to a placebo, as responders or non-responders. We inferred a contribution of endogenous opioids to placebo-induced analgesia from the observation that naloxone produced a greater increase in pain in placebo responders than in non-responders. In a study in which the natural-history group received a hidden infusion of naloxone, Gracely *et al.*² found that naloxone increased the pain in this control group, and concluded that naloxone had antagonized, not a placebo-induced analgesia, but a stress-induced, endorphin-mediated

analgesia occurring after surgery. Based on this conclusion, naloxone should also have significantly increased pain in their placebo group, but this was not observed. It is thus presently not possible to explain differences between our studies and that of Gracely and colleagues.

Importantly, this study also demonstrates that placebo-induced analgesia may be elicited by subtle cues of which a patient or experimental subject is unaware. Therefore, in studies where it is necessary to control for placebo effects, machine infusion of vehicle will allow the experimenters to preserve the same expectation of a therapeutic outcome for both experimental and control subjects.

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- Levine, J. D., Gordon, N. C., Bornstein, J. & Fields, H. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3528-3531 (1979).
- Gracely, R. H., Dubner, R., Wolskee, P. J. & Deeter, W. R. *Nature* **306**, 264-265 (1983).
- Levine, J. D., Gordon, N. C., Jones, R. T. & Fields, H. L. *Nature* **272**, 826-827 (1978).
- Levine, J. D., Gordon, N. C. & Fields, H. L. *Nature* **278**, 740-741 (1979).
- Keppel, G. *Design and Analysis: A Researcher's Handbook* (Prentice-Hall, Englewood Cliffs, 1973).

- Winer, B. J. *Statistical Principles in Experimental Design* 2nd edn (McGraw-Hill, New York, 1971).
- Levine, J. D., Gordon, N. C., Smith, R. & Fields, H. L. *Pain* **10**, 379-389 (1981).
- Jaffe, J. H. & Martin, W. R. in *The Pharmacological Basis of Therapeutics* 6th edn (eds Gilman, A. G., Goodman, L. S. & Gilman, A.) 494-534 (Macmillan, London, 1980).
- Levine, J. D., Gordon, N. C. & Fields, H. L. *Lancet* **ii**, 654-657 (1978).
- Aitken, R. C. B. *Proc. R. Soc. Med.* **92**, 989-992 (1969).
- Scott, J. & Huskisson, E. C. *Pain* **2**, 175-184 (1976).

Inhibition of aldosterone production in the adrenal glomerulosa by atrial natriuretic factor

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Several forms of the polypeptide atrial natriuretic factor (ANF) have been isolated recently from rat and human atria and identified¹⁻³; they are probably associated with the secretory granules of atrial tissue⁴. The potent ability of ANFs to increase urine sodium content is mediated by their direct action on the kidney^{5,6}. We report here the high intrinsic activity of a synthetic replicate of one form of this molecule, ANF(8-33) (ref. 7), to inhibit directly basal aldosterone secretion and its ability to antagonize the stimulatory effects of adrenocorticotropin (ACTH) and angiotensin II (AN-II) on the secretion of aldosterone by rat adrenoglomerulosa cells *in vitro*. Our results suggest that ANF is of clinical importance in the management of aldosterone-dependent hypertension by modifying the adrenocortical response to endogenous ACTH and AN-II.

Rat adrenoglomerulosa cells were obtained by enzymatic digest of adrenals collected from peripubertal male Sprague-Dawley rats (Holtzman, 125 g) using methods described previously⁸⁻¹⁰ (see Fig. 1). Synthetic ANF(8-33) has dramatic effects on the basal secretion of aldosterone by rat glomerulosa cells (Fig. 1). In concentrations as low as $\leq 10^{-9}$ M, there was a significant ($P < 0.01$) inhibition of aldosterone synthesis, even more accentuated at the higher concentrations tested ($P < 0.001$). Similar inhibitory effects on aldosterone formation were obtained when its secretion by the glomerulosa cells *in vitro* was stimulated with either ACTH (Fig. 2a) or AN-II (Fig. 2b). In each case, the simultaneous addition of equimolar amounts of ACTH or AN-II and ANF(8-33) resulted in a significant inhibition of aldosterone secretion ($P < 0.001$) when compared with that obtained by ACTH or AN-II alone. In these samples, corticosterone formation was inhibited by 10^{-7} M ANF(8-33), resulting in decreases from 24.7 ± 0.8 to 7.8 ± 3 ng ml⁻¹ ($P < 0.001$) in basal corticosterone formation, 37.2 ± 1.4 to $21.9 \pm$

0.9 ng ml⁻¹ ($P < 0.001$) on AN-II-stimulated corticosterone formation and 43.4 ± 0.7 to 36.1 ± 0.7 ng ml⁻¹ ($P < 0.001$) in ACTH-stimulated corticosterone formation, suggesting that ANF acts early in the steps of steroidogenesis, possibly as early as, or even before, cholesterol side-chain cleavage. The effect of ANF has also been demonstrated under various experimental designs, including increasing amounts of stimulus with ACTH and/or AN-II (results not shown). The specificity of ANF action on adrenal glomerulosa cells is demonstrated by its inability to modify basal or ACTH-stimulated corticosterone synthesis by rat fasciculata cells (inner zone after decapsulation) in suspension (Table 1). In similar studies using bovine fasciculata-reticularis cells¹¹ ANF(8-33) did not modify basal or ACTH-stimulated cortisol production. The amounts of cortisol formed by cells treated with 10^{-9} M ACTH(1-39) (40.8 ± 1.3 ng per well, $n = 12$) were not different from those formed by cells incubated with ACTH (10^{-9} M) and ANF(8-33) (10^{-9} M) (44.4 ± 1.1 ng per well, $n = 12$). Basal cortisol formation (0.12 ± 0.01 ng per well, $n = 12$) was also unaffected by ANF(8-33) (10^{-9} M) treatment (0.10 ± 0.01 ng per well, $n = 12$). In other experiments ANF(8-33) had no effect on pituitary ACTH secretion, demonstrating clearly a specific locus of action on the adrenal glomerulosa.

The observation that a synthetic replicate of ANF has the intrinsic activity to modify directly, at the glomerulosa cell level, the pattern of aldosterone secretion provides the first evidence for a humoral link between the heart and the adrenal cortex. While somatostatin^{12,13}, β -endorphin¹⁴ and met-enkephalin¹⁵ have been associated with similar biological activities, their physiological significance is uncertain. The potency of ANF

Table 1 Specificity of the effect of ANF(8-33) on adrenal steroidogenesis

Treatment	Corticosterone (ng ml ⁻¹)
Control	81 ± 6
ANF(8-33)	91 ± 8
ACTH(1-39)	782 ± 27
ANF(8-33) + ACTH(1-39)	752 ± 16

Enzymatic digests of cells were prepared from the inner zone of decapsulated adrenals obtained from normal male (125 g) rats, as described in Fig. 1 legend. All treatments were added at a concentration of 10^{-9} M.

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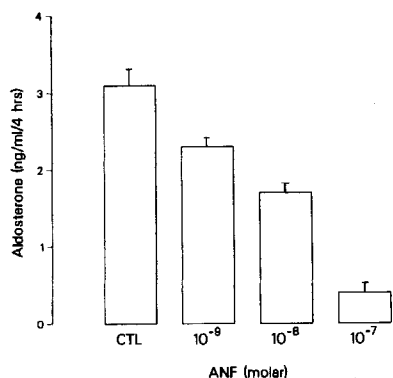


Fig. 1 Effect of ANF(8-33) on basal aldosterone secretion. Rat glomerulosa cells were prepared by enzymatic digestion of 20 rat adrenals after enucleation. The cells remaining on the capsule were digested for 30 min with a mixture of collagenase and DNase (4 mg ml^{-1} , $4 \mu\text{g ml}^{-1}$) for 30 min. Dispersed cells were filtered through gauze and centrifuged at 800 r.p.m. for 15 min. The pellet was resuspended in M199 buffer containing 0.1% bovine serum albumin (BSA) and the cells centrifuged at 800 r.p.m. for 15 min. The cell pellet was again resuspended in M199-0.1% BSA buffer and distributed in 900- μl aliquots to 12 $\times$ 75 plastic tubes. The samples were preincubated for 90 min in a 37 °C waterbath under an atmosphere of 5% CO_2 /95% O_2 . Aliquots of the test samples were added in a 100 μl volume and incubated for 4 h. Aldosterone and corticosterone were measured by radioimmunoassay using antisera purchased from Endocrine Sciences, Oxnard, California, and ^3H -labelled steroid from NEN. Results are the mean $\pm$ s.e.m. of seven replicates. Statistical analysis was performed by analysis of variance and all points are significant ($P < 0.01$) from control. (ANF(8-33) was the gift of Drs R. Hirschmann and D. F. Veber of Merck, Sharp and Dohme Research Laboratories).

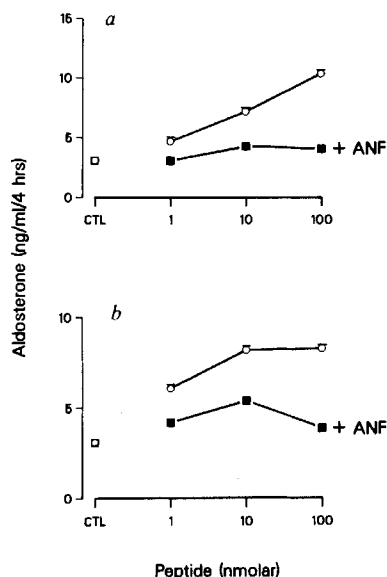


Fig. 2 Effect of ANF(8-33) on stimulated aldosterone secretion. *a*, Rat glomerulosa cells were prepared as described in Fig. 1 and incubated with synthetic human ACTH either alone (open circles) or in combination with equimolar amounts of ANF(8-33) (closed squares). Aldosterone secretion was measured as above by radioimmunoassay. *b*, Cells were incubated with synthetic angiotensin-II (AN-II) either alone (open circles) or in the presence of equimolar amounts of ANF(8-33) (closed squares). Control cells received neither peptide, thereby indicating the ability of ANF to decrease aldosterone production to basal levels. Results are the mean $\pm$ s.e.m. of 7 replicates and all points are significant when compared with their respective control ($P < 0.001$). Synthetic hACTH(1-39) and angiotensin II were synthesized by Dr Nicholas Ling by solid-phase methodology.

(8-33) as a natriuretic hormone, and now in inhibiting basal and stimulated aldosterone formation, suggests that its biological activities are an integral part of the homeostatic mechanisms regulating sodium retention. Furthermore, unlike somatostatin, its inhibitory effect is not restricted to angiotensin-stimulated aldosterone secretion, but affects the formation of both basal and stimulated mineralocorticoids. Moreover, at no point was ANF(8-33) observed to stimulate aldosterone. The observations reported here provide the groundwork for defining the mechanisms by which atrial-derived peptides affect sodium retention and suggest that this peptide may be responsible for the attenuated effects of AN-II on the adrenal cortex during sodium loading^{16,17}. The understanding of some clinical forms of idiopathic hypo- and hypertension^{18,19} may therefore result from defining the interactions between ANF, the adrenal cortex and the basic mechanisms regulating ANF secretion.

After submission of this manuscript, Chartier *et al.*²⁰ and DeLean *et al.*²¹ reported findings similar to those reported here.

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1. Flynn, T. G., deBold, M. L. & deBold, A. J. *Biochem. biophys. Res. Commun.* **117**, 859-865 (1983).
2. Currie, M. G. *et al. Science* **223**, 67-69 (1984).
3. Kangawa, K. & Matsuo, H. *Biochem. biophys. Res. Commun.* **118**, 131-139 (1984).
4. Jamieson, J. D. & Palade, G. E. *J. Cell Biol.* **23**, 151-171 (1964).
5. Sonneberg, H., Cupples, W. A., deBold, A. J. & Veress, A. T. *Can. J. Physiol. Pharmacol.* **60**, 1149-1152 (1982).
6. Keeler, R. *Can. J. Physiol. Pharmacol.* **60**, 1078-1082 (1982).
7. Thibault, G. *et al. FEBS Lett.* **167**, 352-356 (1984).
8. Tait, S. A. S. *et al. Biochemistry* **5**, 775-787 (1974).
9. Edwards, J. *et al. Clin. Endocrinol.* **13**, 45-50 (1980).
10. Douglas, J., Aguilera, G., Kondo, T. & Catt, K. J. *Endocrinology* **102**, 685-696 (1978).
11. Hornsby, P. J. & Gill, G. N. *Endocrinology* **102**, 926-936 (1978).
12. Aguilera, G., Harwood, J. P. & Catt, K. J. *Nature* **292**, 262-263 (1981).
13. Boscaro, M., Scaroni, V., Edwards, C. R. W. & Mantero, F. *J. endocr. Invest.* **5**, 173-177 (1982).
14. Szalas, K. S. & Stark, E. *Life Sci.* **29**, 1355-1361 (1981).
15. Racz, K. *et al. J. clin. Endocr. Metab.* **54**, 656-660 (1982).
16. Nicholls, M. G. *et al. Endocrinology* **102**, 485-493 (1978).
17. Cowley, A. W. & McCaa, R. E. *Circulation Res.* **39**, 788-797 (1976).
18. New, M. I. & Levine, L. S. *Endocrine Rev.* **1**, 421-430 (1980).
19. Zipser, R. D. *et al. J. clin. Endocr. Metab.* **53**, 867-873 (1981).
20. Chartier, L., Schiffrin, E., Thibault, G. & Garcia, R. *Endocrinology* (in the press).
21. DeLean, A. *et al. Endocrinology* (in the press).

Molecular cloning of lymphadenopathy-associated virus

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Lymphadenopathy-associated virus (LAV) is a human retrovirus first isolated from a homosexual patient with lymphadenopathy syndrome, frequently a prodrome or a benign form of acquired immune deficiency syndrome (AIDS)². Other LAV isolates have subsequently been recovered from patients with AIDS or pre-AIDS³⁻⁵ and all available data are consistent with the virus being the causative agent of AIDS. The virus is propagated on activated T lymphocytes and has a tropism for the T-cell subset OKT4 (ref.

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6), in which it induces a cytopathic effect. The major core protein of LAV is antigenically unrelated to other known retroviral antigens^{1,2,7}. LAV-like viruses have more recently been independently isolated from patients with AIDS and pre-AIDS. These viruses, called human T-cell leukaemia/lymphoma virus type III (HTLV-III)⁸⁻¹¹ and AIDS-associated retrovirus (ARV)¹², seem to have many characteristics in common with LAV and probably represent independent isolates of the LAV prototype. We have sought to characterize LAV by the molecular cloning of its genome. A cloned LAV complementary DNA was used to screen a library of recombinant phages constructed from the genomic DNA of LAV-infected T lymphocytes. Two families of clones were characterized which differ in a restriction site. The viral genome is longer than any other human retroviral genome (9.1–9.2 kilobases).

The cDNA first-strand of LAV was synthesized in an endogenous, detergent-activated reaction. LAV virions were purified from the supernatant of FR8 cells, a B-lymphoblastoid LAV-producing line¹³, and the reaction was primed with oligo(dT). Three cDNA clones, pLAV13, 75 and 82, carrying inserts of 2.5, 0.6 and 0.8 kilobases (kb), respectively, were characterized further (Fig. 1). All three inserts have a common restriction pattern at one end, indicative of a common priming site. The 50-base pair (bp) common *Hind*III–*Pst*I fragment was sequenced and shown to contain an oligo(dA) stretch preceding

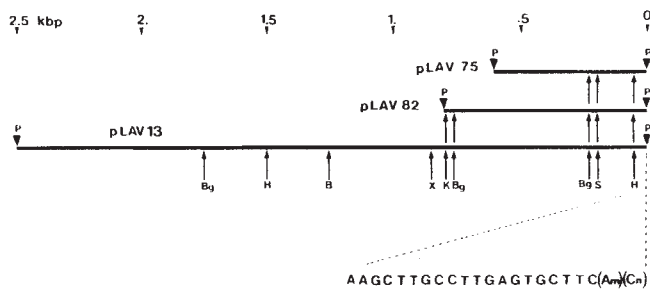


Fig. 1 Restriction maps of cDNA clones derived from LAV genomic RNA. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; K, *Kpn*I; S, *Sac*I; X, *Xho*I.

Methods: LAV cDNA was synthesized in an endogenous detergent-activated reaction. For each reaction, LAV virions were purified on a 20–60% sucrose gradient as described previously¹, from 200 ml of supernatant of the LAV-producing FR8 line¹³. Virus-containing fractions were pooled, diluted with NTE buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA) and centrifuged (Beckman type SW56 rotor, 50,000 r.p.m., 60 min). The viral pellet was resuspended in 250 μ l of NTE. Reaction volume was adjusted to 1 ml and final concentrations were: 50 mM Tris-HCl pH 7.8, 25 mM NaCl, 6 mM MgCl₂, 10 mM dithiothreitol, 0.02% Triton X-100, 0.1 mM of each of dATP, dGTP, TTP, 4 μ M dCTP including 200 μ Ci of [α -³²P]dCTP (400 Ci mmol⁻¹, Amersham) and 50 μ g ml⁻¹ oligo(dT) primer. Incubation was at 37 °C. After 15 min, dCTP was added to 25 μ M. At 45 min, the reaction was stopped with EDTA and SDS (final concentrations 20 mM and 0.5%, respectively). After 1 h of proteinase K digestion (100 μ g ml⁻¹, 37 °C), the reaction mixture was extracted with phenol/chloroform and cDNA–RNA hybrids were ethanol-precipitated. Second-strand synthesis with nuclease-free DNA polymerase I (Boehringer) and RNase H (BRL) and dC-tailing with terminal transferase (Boehringer) were performed according to Gubler and Hoffman²⁶. Tailed double-stranded cDNA was annealed to dG-tailed *Pst*I-linearized pBR327 vector. *Escherichia coli* C600 recBC was transformed by the CaCl₂ method; 500 recombinant clones were screened *in situ*²⁷ with a ³²P-labelled LAV cDNA in which the first strand had been synthesized as described above, except that an alkaline hydrolysis step was included. Approximately 10% of recombinants proved positive, the majority of which formed a family of cross-hybridizing clones. Three recombinants, pLAV13, pLAV75 and pLAV82, carrying inserts of 2.5, 0.6 and 0.8 kb, respectively, were analysed further. There are no sites for *Eco*RI, *Nru*I, *Pvu*I, *Sal*I, *Sma*I, *Stu*I or *Xba*I in the pLAV13 insert. The *Hind*III–*Pst*I fragment was subcloned into M13mp8 and sequenced according to Sanger *et al.*²⁸ using a 15-mer primer (Biolabs) and [α -³²P]dCTP (Amersham).

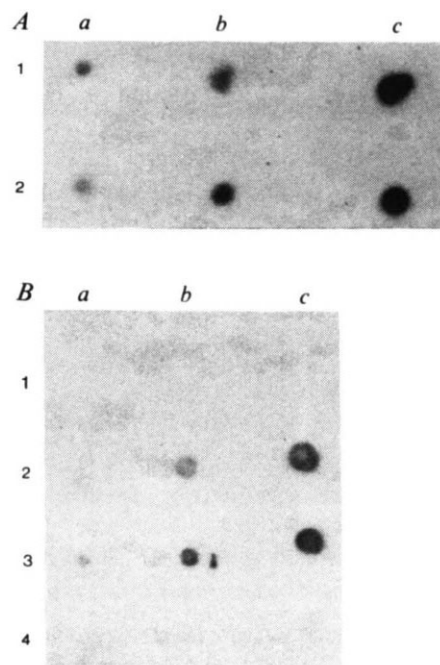


Fig. 2 Rapid dot-blot technique for LAV detection in cell culture supernatant. Spots represent: A, a, 1 μ l; b, 2 μ l; c, 4 μ l of concentrated (250 $\times$) cell culture supernatant from (1) LAV-producing CEM cells (reverse transcriptase activity (RT), determined as described previously¹, was 140,000 c.p.m. ml⁻¹); (2) LAV-producing Epstein-Barr-transformed B-cell line FR8 (RT 175,000 c.p.m. ml⁻¹). B, a, 1 μ l; b, 2 μ l; c, 5 μ l of 100 $\times$ concentrated supernatant from (1) uninfected normal T lymphocytes (no RT activity); (2) LAV-producing normal T lymphocytes (RT 170,000 c.p.m.); (3) LAV-producing CEM line (RT 150,000 c.p.m.); and (4) culture of bone marrow lymphocytes from a haemophiliac patient⁴ with AIDS (RT 7,000 c.p.m.).

Methods: Cell culture supernatants were pelleted through 0.5 ml 20% sucrose cushions in NTE buffer (Beckman type SW56 rotor, 50,000 r.p.m., 1 h, 4 °C). The pellet was resuspended in NTE buffer as indicated. Concentrated virus was spotted onto dried nylon filters (Zetabind) presoaked in 20 $\times$ SSC (3 M NaCl, 0.3 M sodium citrate). After baking (at least 30 min at 80 °C), filters were hybridized with ³²P nick-translated pLAV13 insert (Fig. 1) (specific activity >10⁸ c.p.m. per μ g) for 12–16 h in stringent conditions (50% formamide, 5 $\times$ SSC, 42 °C), washed (0.1 $\times$ SSC, 0.1% SDS, 65 °C, 2 $\times$ 30 min), and exposed for 20 h (Kodak XAR5 film with an intensifying screen) at –70 °C.

the cloning dC tail. The clones are thus copies of the 3' end of a poly(A) RNA.

The specificity of pLAV13 was determined in a series of filter hybridization experiments using nick-translated pLAV13 insert as a probe. First, using an adapted spot-blot technique, we could detect LAV virion RNA from normal T cells, FR8 and other B-cell lines and CEM cells (L.M. and R. Weiss, unpublished results; Fig. 2). LAV was also detected in a bone marrow cell culture (Fig. 2B, line 4) from a haemophiliac with AIDS⁴, in spite of the low titre of virus in the supernatant. Uninfected cultures proved negative (Fig. 2B, line 1). Second, the probe detected DNA in the Southern blots of LAV-infected T lymphocytes and CEM cells (Fig. 3). No hybridization was detected in DNA from uninfected lymphocytes or from normal liver (data not shown) in the same hybridization conditions. A characteristic 1.45-kb *Hind*III fragment which co-migrated with an internal viral fragment in *Hind*III-cleaved pLAV13 (Fig. 1) was detected in the Southern blots. Bands at 2.3 and 6.7 kb were also detected. Together, these data show that pLAV13 DNA is exogenous to the human genome and detects both RNA and integrated DNA forms derived from LAV-infected cells. Thus, pLAV13 is LAV specific. Being oligo(dT)-primed, pLAV13 must contain the R and U3 regions of the long terminal repeat (LTR) as well as

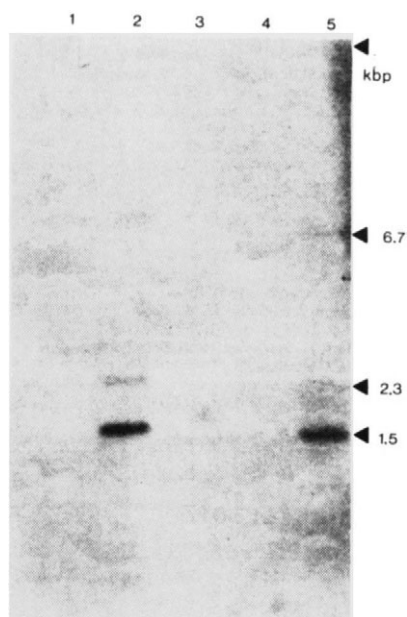


Fig. 3 Southern blot of *Hind*III-restricted genomic DNA from LAV-infected and uninfected cells hybridized with pLAV13. *Hind*III-restricted high relative molecular mass DNA from: lane 1, uninfected CEM cells; lane 2, LAV-infected CEM cells; lane 3, uninfected T cells after 5 days' culture; lane 4, LAV-infected T cells 2 days after infection; lane 5, LAV-infected T cells 5 days after infection.

Methods: Peripheral blood T lymphocytes of a healthy donor were stimulated for 3 days with phytohaemagglutinin, after which they were infected with LAV (isolate BRU-LAV1) at 10^4 c.p.m. reverse transcriptase activity per 10^6 cells as described previously¹, except for part of the culture kept uninfected for controls. Two and five days after infection, genomic DNA was extracted. *Hind*III-digested DNA ($10 \mu\text{g}$) was electrophoresed through a 0.8% agarose gel and Southern blotted. The filter was hybridized in 10 ml of 50% formamide, $5\times\text{SSC}$, $1\times\text{Denhardt's}$, 10% dextran sulphate with $100 \mu\text{g ml}^{-1}$ denatured sonicated salmon sperm DNA and 2×10^7 c.p.m. of nick-translated pLAV13 insert (4×10^8 c.p.m. per μg) for 10 h at 42°C . The filter was washed at 68°C in $0.1\times\text{SSC}$, 0.1% SDS for 2×30 min and exposed to Kodak XAR5 film at -70°C for 16 h using an intensifying screen.

the 3' end of the coding region, assuming a conventional retroviral genome structure.

Having found a *Hind*III site about 20 bp 5' of the poly(A) stretch and thus within the R region of the LTR, we cloned the LAV genome by making a partial *Hind*III digest of genomic DNA from LAV-infected T cells of a healthy donor. A 9 ± 1.5 -kb DNA-containing fraction was precipitated and ligated into the *Hind*III arms of phage vector $\lambda\text{L47.1}$ (ref. 14). When nick-translated pLAV13 insert was used as a probe to screen $\sim 2\times 10^6$ phage plaques *in situ*, five independent clones were obtained. A restriction map of clone λJ19 and of a *Hind*III variant, λJ81 , are shown in Fig. 4. Recombinants λJ27 , λJ31 and λJ57 have the same *Hind*III map as λJ19 , while λJ81 is so far unique. As the two clones were derived from the first isolate¹ of LAV reported (isolate BRU, or LAV1), we refer to the two viral genomes as LAV1a (λJ19) and LAV1b (λJ81). λJ19 shows four *Hind*III bands of 6.7, 1.45, 0.6 and 0.52 kb, the first two of which correspond to bands in the genomic blot of *Hind*III-restricted DNA (Fig. 3, lane 5). The smallest bands (0.6 and 0.52 kb) were not seen in the genomic blot, but the fact that they appear in all the independently derived clones analysed indicates that they represent internal and not junction fragments, assuming random integration of LAV proviral DNA. However, the 0.52-kb band hybridizes with pLAV13 DNA (Fig. 4) through the small *Hind*III-*Pst*I fragment of pLAV13. Thus, the 0.5-kb *Hind*III fragment of λJ19 contains the R/U5 junction within the LTR. The finding of two small *Hind*III fragments in the 5' region reinforces

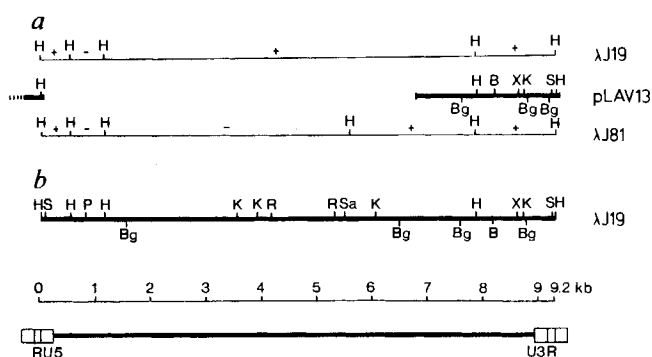


Fig. 4 Restriction maps of LAV proviral DNA in clones λJ19 (LAV1a) and λJ81 (LAV1b). *a*, *Hind*III restriction maps of LAV proviral DNA in clones λJ19 and λJ81 . Those *Hind*III fragments detected by pLAV13 are marked by +, those not, by -. The restriction map of the pLAV13 cDNA clone is also shown. *b*, Restriction map of λJ19 . Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sac*I; Sa, *Sall*; X, *Xho*I. Beneath the scale is a schema for the general structure of retroviruses showing the LTR elements U3, R and U5. Only the R/U5 boundary has been defined (Fig. 1) and other boundaries are drawn only figuratively.

Methods: DNA from LAV-infected T cells was partially digested with *Hind*III and fractionated on a 5–40% sucrose gradient in 10 mM Tris-HCl pH 8, 10 mM EDTA, 1 M NaCl (Beckman type SW41 rotor, 16 h, 40,000 r.p.m.). A single fraction (9 ± 1.5 kb) was precipitated with $20 \mu\text{g ml}^{-1}$ dextran T40 as carrier and taken up in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). $\lambda\text{L47.1}$ (ref. 14) *Hind*III arms were prepared by first ligating the *cos* sites followed by *Hind*III digestion and fractionation through a 5–40% sucrose gradient as above. Fractions containing only the λ *Hind*III arms were pooled, precipitated and taken up in TE buffer. Ligation of arms to DNA was made at $\sim 200 \mu\text{g ml}^{-1}$ DNA using a 3:1 molar excess of arms and 300 U of T4 DNA ligase (Biolabs). *In vitro* packaging lysates were made according to ref. 29. After *in vitro* packaging, the phage lysate was plated out on NM538 or a C600 recBC strain. Approximately 2×10^6 plaques were screened by *in situ* hybridization³⁰ using nitrocellulose filters. Hybridization was performed at 68°C in $1\times\text{Denhardt's}$ solution, 0.5% SDS, $2\times\text{SSC}$, 2 mM EDTA. Probe: ^{32}P nick-translated insert of pLAV13 at $>10^8$ c.p.m. per μg . Filters were washed for 2×30 min in $0.1\times\text{SSC}$ 10.1% SDS at 68°C , and exposed to Kodak XAR-5 film for 24–40 h with intensifying screens at -70°C . Seven positive clones were identified and plaque-purified on a C600 recBC strain. Liquid cultures were grown and the recombinant phages banded in CsCl. Phage DNA was extracted and digested in the appropriate conditions. The restriction maps were orientated by hybridizing blots to pLAV13 DNA, which maps the 3' coding sequences of the viral genome as well as the U3-R region of the LTR. All cloning and amplification of LAV genomic clones was carried out in a P3 laboratory.

the usefulness of cloning LAV by partial restriction of genomic DNA.

λJ81 seems to be a restriction site polymorph of λJ19 , showing five *Hind*III bands of 4.3, 2.3, 1.45, 0.6 and 0.52 kb (Fig. 4). The 2.3-kb band is readily detected in the genomic blot by a pLAV13 probe, although the 4.3-kb fragment is not. The finding that nick-translated λJ19 DNA hybridizes to all five *Hind*III bands of λJ81 in stringent hybridization and washing conditions indicates that λJ81 is a *Hind*III variant and not a recombinant virus. Also, other mapped restriction sites in λJ81 are identical to those of λJ19 (not shown). Thus, the *Hind*III restriction pattern in the Southern blot can be explained by variation within the single isolate of LAV used to infect the T cells.

HTLV-I¹⁵ and HTLV-II¹⁶ constitute a pair of C-type transforming retroviruses with a tropism for the T-cell subset, OKT4. Both genomes (comprising one LTR) are ~ 8.3 kb long^{17,18}, have an X region and show extensive sequence homology. They hybridize between themselves in reasonably stringent conditions (40% formamide, $5\times\text{SSC}$) and the X regions hybridize even at 60% formamide¹⁹. Thus, a conserved X region is a hallmark of

this class of virus. We have compared cloned LAV DNA and cloned HTLV-II DNA (pMO)²⁰ by blot-hybridization and find no cross-hybridization in low stringency conditions of hybridization and washing ($T_m = 55^\circ\text{C}$), even after 2 days' exposure at -70°C using intensifying screens (data not shown).

The human T-lymphotropic retroviruses HTLV-III⁸ and ARV¹², recently isolated from patients with AIDS or pre-AIDS, have similar morphological, biochemical and immunological properties to LAV, which suggests that they probably represent different isolates of the LAV prototype. DNA hybridization between HTLV-III and HTLV-I and -II has been reported, most noticeably at the *gag-pol* junction and less so in the characteristic X region of HTLV-I and -II²¹. As mentioned above, we could detect no such hybridization and conclude that the reported homology must have been due to either (1) the use of an uncloned cDNA as hybridization probe, (2) the fact that the isolates in question differ substantially from those we have cloned, or (3) the possibility that HTLV-III and a HTLV-I/II-like virus were co-infecting the cells. The last possibility may also apply to the preliminary report of cross-hybridization between a LAV-like virus and a cloned HTLV-II DNA probe⁷. Thus, we find no molecular evidence of a relationship between LAV and HTLV. Furthermore, the LAV genome is ~9 kb long, compared with 8.3 kb for the HTLV viruses^{17,18}. Despite their comparable genome sizes, LAV does not cross-hybridize with Visna virus²² (~9 kb) (data not shown) or with several human endogenous viral genomes (ref. 23 and M. Martin, personal communication) in non-stringent conditions ($T_m = 55^\circ\text{C}$). These data and morphological and immunological dissimilarities^{1,2} between LAV and the HTLV-I/-II pair all point to LAV being a novel class of human retrovirus.

In conclusion, we have molecularly cloned the complete genome of LAV from freshly infected activated T cells of a healthy donor. It has been shown that the tropism of certain retroviruses resides in the LTR^{24,25} and that sequence differences and insertions/deletions are present in the LTRs of leukaemogenic and non-leukaemogenic retroviruses. It is thus possible that LAV and LAV-like viruses passaged through B- and T-transformed cell lines^{8,12,13} might have undergone some attenuation. Although the cDNA clones were made from a LAV-producing B-cell line, the genomic clones were isolated from LAV-infected normal T cells. Thus, the clones represent LAV genomes that have not been selected or adapted to a particular cell line. However, the LAV genome is shown to be polymorphic even within a single isolate and independent isolates will probably differ widely.

The availability of cloned LAV DNA should facilitate the understanding of the molecular mechanism of viral replication, and the tropism of the virus. The DNA sequence of LAV opens up the possibility of expressing the viral *gag* and *env* gene products and of studying the molecular basis of LAV antigenicity.

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1. Barré-Sinoussi, F. *et al. Science* **220**, 868-871 (1983).
2. Pinching, A. J. *Clin. exp. Immun.* **56**, 1-13 (1984).
3. Montagnier, L. *et al. in Human T-cell Leukemia Viruses* (eds Gallo, R. C., Essex, M. & Gross, L.) 363-379 (Cold Spring Harbor, New York, 1984).
4. Vilmer, E. *et al. Lancet* **ii**, 753-757 (1984).
5. Feorino, M. P. *et al. Science* **225**, 69-72 (1984).
6. Klatzmann, D. *et al. Science* **225**, 59-63 (1984).
7. Kalyanaraman, V. S. *et al. Science* **225**, 321-323 (1984).
8. Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. *Science* **224**, 497-500 (1984).
9. Gallo, R. C. *et al. Science* **224**, 500-503 (1984).
10. Schüpbach, J. *et al. Science* **224**, 503-505 (1984).
11. Sarngadharan, M. G., Popovic, M., Bruch, L., Schüpbach, J. & Gallo, R. C. *Science* **224**, 506-508 (1984).

12. Levy, J. A. *et al. Science* **225**, 840-842 (1984).
13. Montagnier, L. *et al. Science* **225**, 63-66 (1984).
14. Loenen, W. A. M. & Brammar, W. J. *Gene* **10**, 249-259 (1980).
15. Gallo, R. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 5680-5683 (1982).
16. Kalyanaraman, V. S. *et al. Science* **218**, 571-573 (1982).
17. Seiki, M., Hattori, S., Hirayama, Y. & Yoshida, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3618-3622 (1983).
18. Chen, I. S. Y., McLaughlin, J., Gasson, J. C., Clark, S. C. & Golde, D. W. *Nature* **305**, 502-505 (1983).
19. Shaw, G. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 4544-4548 (1984).
20. Gelmann, E. P., Franchini, G., Manzari, V., Wong-Staal, F. & Gallo, R. C. *Proc. natn. Acad. Sci. U.S.A.* **81**, 993-997 (1984).
21. Arya, S. K. *et al. Science* **225**, 927-930 (1984).
22. Harris, J. D. *et al. Virology* **113**, 573-583 (1981).
23. Steele, P. E., Rabson, A. B., Bryan, T. & Martin, M. A. *Science* **225**, 943-947 (1984).
24. Lenz, J. *et al. Nature* **308**, 467-470 (1984).
25. Chen, I. S. Y., McLaughlin, J. & Golde, D. W. *Nature* **309**, 276-279 (1984).
26. Gubler, U. & Hoffman, B. J. *Gene* **25**, 263-269 (1983).
27. Grunstein, M. & Hogness, D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3961-3965 (1975).
28. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5463-5476 (1977).
29. Ish-Horowitz, D. & Burke, J. F. *Nucleic Acids Res.* **9**, 2989-2998 (1981).
30. Benton, W. D. & Davis, R. W. *Science* **196**, 180-182 (1977).

Molecular cloning of AIDS-associated retrovirus

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Retroviruses cause a wide variety of diseases in avian and mammalian species. Human acquired immune deficiency syndrome (AIDS) leads to collapse of the immune system and death by a wide variety of opportunistic infections; unusual forms of cancer are associated with this syndrome. Retroviruses have been recovered from tissues of AIDS patients and from patients with related conditions. These similar newly-isolated viruses are lymphadenopathy-associated virus (LAV)¹, human T-cell lymphotropic virus (HTLV-III)^{2,3} and AIDS-associated retrovirus (ARV-2)⁴. We have identified a RNA genome of ~9 kilobases (kb) in virions purified from the culture medium of a human T-cell tumour line infected with ARV-2. A cDNA probe made from viral RNA detected circular DNA molecules and proviral forms in infected cells. We prepared a library of infected cell DNA. Recombinant phage included those with a 9.5-kb proviral DNA and viral DNA permuted with respect to the single *EcoRI* site. Comparison of three ARV isolates from different AIDS patients revealed polymorphism of restriction endonuclease sites.

HUT-78 cells, originating from a human T-cell lymphoid tumour⁵, were used to propagate the ARV-2 strain of virus⁴. To characterize the viral genome, RNA was extracted from purified virions and electrophoresed on agarose gels containing methyl mercury hydroxide⁶. A distinct ~9-kb RNA species was observed (Fig. 1) with smaller heterogeneous RNA and some ribosomal RNA species. The 9-kb RNA species was used as a template with random primers in a reverse transcriptase reaction to produce a virus-specific cDNA probe⁷. RNA of virus obtained from cells infected with ARV-2 or with two additional isolates, ARV-3 and ARV-4, showed distinct bands at 9 kb that hybridized with the cDNA probe (Fig. 1).

With this cDNA probe, we examined the structure of viral DNA in infected cells by digestion with restriction enzymes, electrophoresis in agarose gels and Southern blotting. No specific bands were detected in several digests of DNA from uninfected cells (Fig. 2a, lanes C, E), whereas bands were seen in infected cells (Fig. 2a, lane A). Undigested DNA from infected cells contained a species at 5.5 kb, a faint species at 6 kb

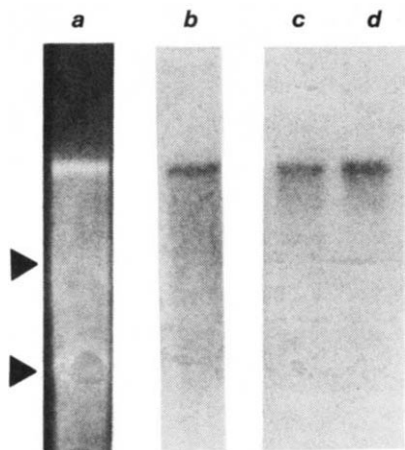


Fig. 1 Analysis of RNA from ARV. To produce an acute infection, the HUT-78 cells were inoculated with virus and fresh medium was added to maintain growing cultures at intervals of 2–3 days. Virus production was monitored by measuring reverse transcriptase activity with exogenous template in viral particles released into the culture medium⁴. After inoculation with tissue culture virus, progeny virus was detected at 3–4 days after infection and production peaked at 6–8 days. To purify virus particles, the culture medium was spun at low speed (1,000 r.p.m., 5 min, 4 °C; all subsequent operations were at 4 °C) to remove cells and further clarified by centrifugation at 7,000 r.p.m. for 10 min. This supernatant was centrifuged at 26,000 r.p.m. for 1 h in the SW28 rotor to pellet virus. The pellet from 100 ml culture medium was resuspended in 2 ml 10 mM Tris-HCl, pH 7.5. The resuspended virus pellet was treated with 10 $\mu\text{g ml}^{-1}$ DNase (Boehringer-Mannheim) for 1 min on ice and layered onto a 10–50% sucrose gradient (buffer containing 100 mM NaCl, 0.1 mM EDTA and 20 mM Tris-HCl, pH 7.6) and centrifuged in the SW41 rotor for 2 h at 34,000 r.p.m. Fractions were collected, extracted with phenol, and nucleic acid from the aqueous phase was adjusted to 300 mM NaCl and precipitated with cold ethanol. A sample of each fraction was electrophoresed on 1% agarose gels (containing 5 mM methyl mercury hydroxide)⁶ and stained with ethidium bromide to locate the fraction enriched for virion RNA (lane a). Arrows show the positions of ribosomal RNA markers at 4.9 and 1.9 kb. The remaining RNA from this gradient fraction was electrophoresed in low-melting 1% agarose gel (containing 5 mM methyl mercury hydroxide). The region in the gel containing the ~9 kb RNA species was cut out, melted at 70 °C, and the RNA extracted with phenol and concentrated by precipitation with cold ethanol. We obtained 0.5–1 μg of purified 9 kb RNA from 100 ml of culture medium. To synthesize a radioactive cDNA probe, this RNA was used as template for reverse transcriptase with random DNA primers (calf thymus⁷) and with the addition of 20 $\mu\text{g ml}^{-1}$ actinomycin D to the reaction. RNA from ARV-2, ARV-3 and ARV-4, electrophoresed on 1% agarose gels (containing methyl mercury hydroxide), were transferred to nitrocellulose filters⁹, and hybridized with the cDNA probe (lanes b, c and d, respectively). These hybridizations were performed in 50% formamide, 3 $\times$ SSC at 42 °C and washes were at 50 °C in 0.2 $\times$ SSC.

and a broad band at the exclusion limit of the gel (>15 kb). We suggest that the DNA species at 5.5 and 6 kb represent unintegrated viral DNA in a circular configuration containing, respectively, one and two long terminal repeats (LTRs); the upper broad band (>15 kb) represents proviruses integrated into host cell DNA (Fig. 2a, lane A).

EcoRI digestion gave a 9.5-kb fragment, and *SacI* gave 5.7- and 3.8-kb fragments (Fig. 2a, lane D). Digestion of infected cell DNA with a mixture of *EcoRI* and *SacI* yielded DNA fragments at 5.4 and 3.8 kb (Fig. 2a, lane F). Digestions with *HindIII* (Fig. 4, *PvuII* or *BglII*) produced five or more DNA fragments (data not shown). The sum of the molecular weights of the viral DNA fragments in each digest was 9.2–9.5 kb. A restriction enzyme map of a proviral form in HUT-78 cells infected with ARV-2 was produced (Fig. 2).

A complex pattern was produced in *KpnI* digestions. A major species of 5.2 kb and a minor species of 4.2 kb were observed

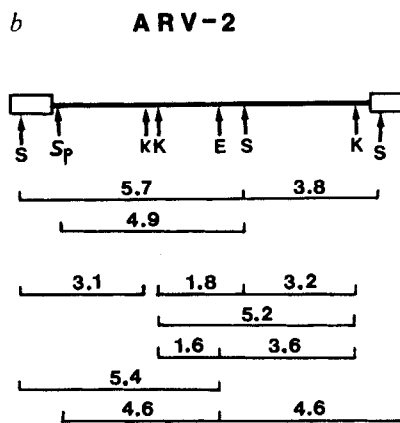
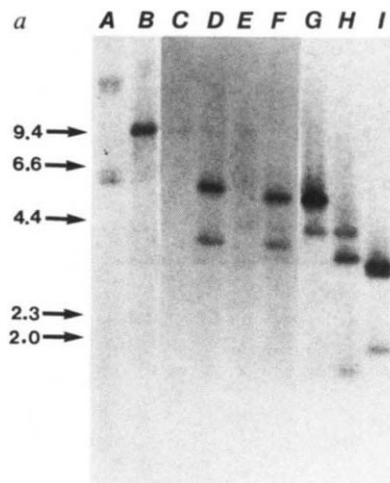


Fig. 2 Detection and organization of ARV DNA in infected cells. Whole-cell DNA was prepared by a phenol extraction method¹⁰ from uninfected HUT-78 cells and from cells 10 days after infection with ARV-2. These DNA preparations were analysed by restriction enzyme digestions, electrophoresis on 1% agarose gels coupled with transfer to nitrocellulose¹⁰ and annealing with radioactive viral cDNA probe⁷. Annealing was done at 42 °C in 50% formamide and 3 $\times$ SSC, and washes were performed at 50 °C in 0.2 $\times$ SSC. **a**, Lane A, undigested infected cell DNA; B, infected cell DNA, *EcoRI*; C, HUT-78 DNA, *SacI*; D, infected cell DNA, *SacI*; E, HUT-78 DNA, *EcoRI* and *SacI*; F, infected cell DNA, *EcoRI* and *SacI*; G, H, I, infected cell DNA digested with, respectively, *KpnI*, *KpnI* and *EcoRI*, and *KpnI* and *SacI*. Markers (in kb) are *HindIII* fragments from bacteriophage λ DNA. **b**, The positions of restriction enzyme sites and DNA fragment sizes. Open boxes, inferred LTR structures; S, *SacI*; E, *EcoRI*; K, *KpnI*; Sp, *SphI* site. The orientation of the map is based on DNA sequence information (our unpublished results).

(Fig. 2a, lane G). Digestion with a mixture of *KpnI* and *EcoRI* cleaved the 5.2-kb fragment to a 3.5 and a 1.7 kb species; the faint 4.2-kb species remained (Fig. 2a, lane H). *SacI* cleaved both of the *KpnI* fragments (Fig. 2a, lane I). These digestion patterns can be explained if the *KpnI*-generated 5.2-kb fragment is an internal proviral DNA fragment and the minor species is a circle junction from circular viral DNA possessing one LTR. Autoradiographs of the *KpnI* digest showed a very faint band at 5.1 kb not affected in the double digestion with *KpnI* and *EcoRI*; this 5.1-kb band disappeared after double digestion with *KpnI* and *SacI* (Fig. 2a, lane I). The 5.1-kb fragment in the *KpnI* digest probably represents the circle junction in unintegrated circular viral DNA containing two LTRs. Circular viral DNA molecules were observed for up to 2 weeks after infection of tissue culture cells; thus either circular viral DNA is stable for long periods of time or the cells in culture are reinfected continually.

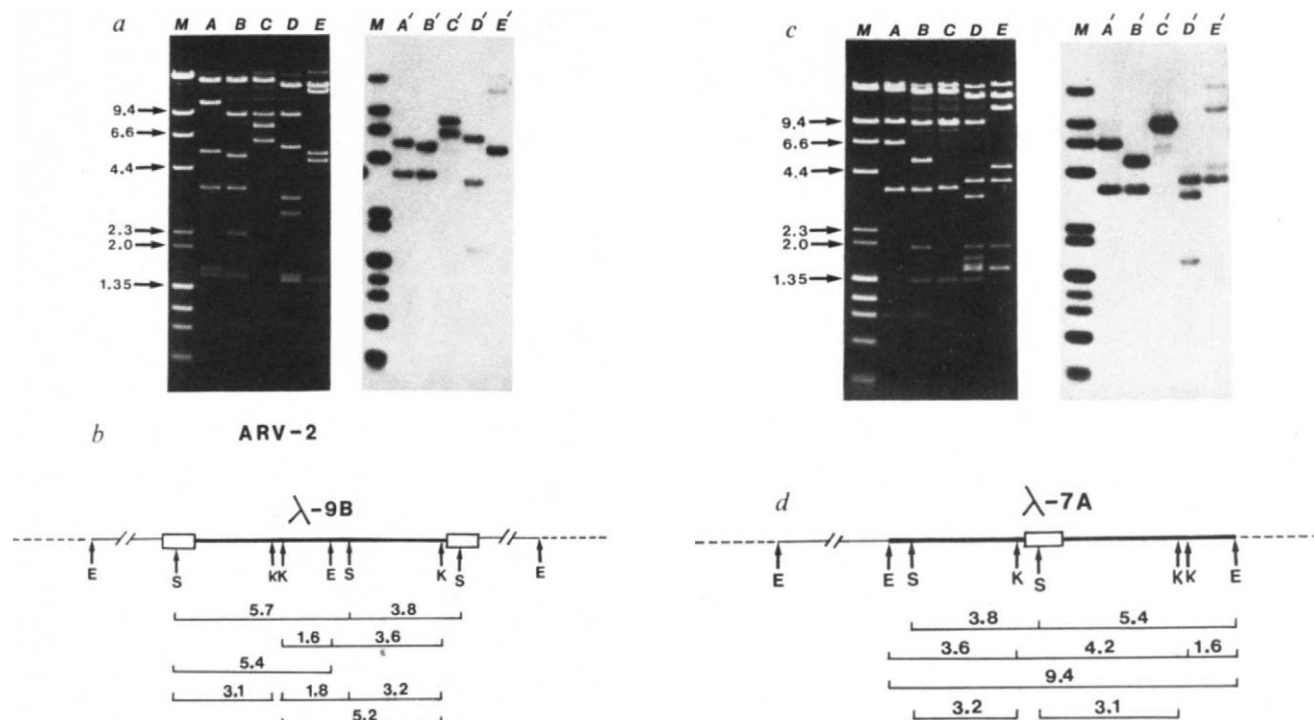


Fig. 3 Structures of recombinant phage containing ARV DNA. *a*, Purified λ -9B phage DNA digested by restriction enzymes; left-hand side, visualized with UV light; right-hand side, DNA transferred to nitrocellulose and annealed with viral cDNA probe. *M*, *Hind*III DNA fragments of phage λ and *Hae*III DNA fragments of phage Φ X174 (in kb). *A*, *A'*, *Sac*I digest; *B*, *B'*, *Eco*I and *Sac*I; *C*, *C'*, *Eco*I and *Sac*I; *D*, *D'*, *Kpn*I and *Eco*I; *E*, *E'*, *Kpn*I. *b*, Structures of recombinant phage DNA λ -9B. Heavy lines, viral DNA; open boxes, inferred LTRs; light lines, flanking-cell DNA; broken lines, phage vector DNA. *c*, As *a* but with λ -7A phage DNA. *d*, As *b* but with λ -7A phage DNA.

Methods: The DNA from HUT-78 cells infected with ARV-2 was treated with *Eco*RI under conditions yielding partial digest products (0.25 unit *Eco*RI per μ g cell DNA, 15 min, 37 °C) and centrifuged on 10–40% sucrose gradients (in buffer containing 0.5 M NaCl, 10 mM EDTA, 20 mM Tris-HCl, pH 7.5) at 35,000 r.p.m. for 12 h at 15 °C. Fractions were collected, a sample of each was electrophoresed on 1% agarose gels and the fractions containing 9–15-kb DNA were pooled. This size-selected DNA was concentrated by precipitation with ethanol and subsequently ligated to EMBL-4 bacteriophage DNA^{11,12} (digested with *Eco*RI, *Bam*HI and *Sal*I). (Packaging extract from Promega Biotech.) DP50 supF was the bacterial host. Plaquing efficiencies with native EMBL-4 vector DNA were about $1 \times 10^8 \mu\text{g}^{-1}$ and the background for the digested vector DNA alone was about $1 \times 10^5 \mu\text{g}^{-1}$. Plaquing efficiency for the ligated DNA (size-selected cell DNA and digested vector DNA) was $3 \times 10^6 \mu\text{g}^{-1}$. Ten 150-mm culture dishes were plated with 50,000 phage per culture, and phage were transferred to nitrocellulose in the double-life procedure¹¹. Viral cDNA probe (Fig. 2) identified recombinant phage plaques containing ARV DNA; these plaques were further purified and monitored with viral cDNA probe through secondary and tertiary platings. Plaque-purified phage containing ARV DNA were propagated in liquid culture in DP50; phage particles were collected and banded in CsCl gradients and recombinant phage DNA was prepared by phenol extraction followed by ethanol precipitation¹². We then digested 1 μ g purified phage DNA with restriction enzymes and electrophoresed them on 1% agarose gels.

To produce substrate for a recombinant DNA library, whole-cell DNA from cells infected with ARV-2 was partially digested with *Eco*RI; 9–15-kb cell DNA was cloned into an EMBL-4 bacteriophage λ vector and recombinant phage were identified with the virus-specific cDNA probe. Two examples of the 11 recombinant phage were selected for detailed description: λ -9B contained an insertion of full-length proviral DNA with flanking cell sequences and λ -7A contained a full-length viral genome permuted with respect to the *Eco*RI site. Viral DNA in each of these recombinant phage is 9.5 kb; the restriction endonuclease digest patterns are the same as those for viral DNA found in infected cells (Fig. 2). In addition to the two types of recombinant phage containing ARV DNA (Fig. 3), we obtained phage with the left half of the viral genome from the *Eco*RI site in viral DNA extending into flanking cell DNA and also phage with the right half of the viral genome from the *Eco*RI site in viral DNA extending into flanking cell DNA (our unpublished results).

To validate the cloned ARV DNA, a radioactive probe was prepared representing the entire cloned genome (see Fig. 4 legend). The cDNA probe (Figs 1, 2) and the probe representing cloned viral DNA anneal to the same DNA species in several restriction enzyme digests of DNA from infected cells (compare lanes *a*–*c* with *d*–*f* in Fig. 4).

We next assessed the relationship among three independent ARV isolates. Restriction enzyme digests of DNA from HUT-78 cells infected with ARV-3 and ARV-4 were analysed with the probe made from cloned ARV-2 DNA. The *Sac*I digests of ARV-3 DNA were similar to that of ARV-2 whereas the *Hind*III digests displayed different patterns. The *Sac*I digest and the *Pst*I digest of ARV-4 DNA differed from the corresponding digests of ARV-2 DNA (Fig. 4). The intensity of the annealing signals obtained with ARV-3 and ARV-4 samples was much lower (about 10-fold less) than that for ARV-2 DNA; this observation probably reflects the smaller number of cells infected in the ARV-3 and ARV-4 cultures or the smaller number of viral DNA molecules in individual cells. The virus-specific DNA fragments produced by *Sac*I treatment of ARV-3 and ARV-4 DNA totalled 9.0–9.5 kb, a value similar to that of ARV-2 and in agreement with the RNA genome size (Fig. 1). The antigenic relatedness of these three viral isolates was demonstrated in immunofluorescence studies with an AIDS reference serum (EW5111, from Dr Paul Feorino, Centers for Disease Control, Atlanta, Georgia). This serum reacted with HUT-78 cells infected with ARV-2 and human peripheral blood lymphocytes infected with ARV-3 and ARV-4 (ref. 4).

We have described here the characterization of the RNA and DNA genomes of ARV. Serological studies suggest that ARV,

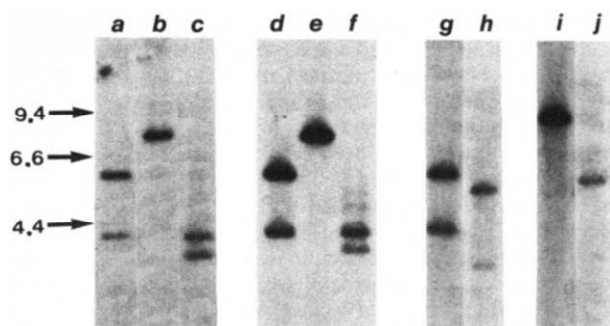


Fig. 4 Detection of ARV DNA in cells infected with three independent isolates of ARV. Whole-cell DNA was prepared from HUT-78 cell cultures infected with ARV-2, ARV-3 and ARV-4 and analysed by digestion with restriction enzymes as described in Fig. 3. DNA from recombinant phage λ -7A was digested with *EcoRI* and the 9.5-kb ARV DNA fragment was cloned into the *EcoRI* site of the vector pUC19. This recombinant plasmid was propagated, digested with *EcoRI* and the viral 9.5-kb DNA fragment was purified by electrophoresis in 1% agarose gels (low-melting agarose). Viral DNA was eluted from the agarose (Fig. 1), denatured by boiling in water for 2 min and used as template for reverse transcriptase with random DNA primers (calf thymus)⁶. Lanes a, b, c, DNA from HUT-78 cells infected with ARV-2 was digested with *SacI*, *PstI* and *HindIII*, respectively; viral cDNA probe was used (Fig. 2). Lanes d, e, f, DNA from HUT-78 cells infected with ARV-2 and digested with *SacI*, *PstI* and *HindIII*, respectively; probe to cloned ARV-2 DNA was used. Lanes g, h, DNA from HUT-78 cells infected with ARV-3, digested with *SacI* and *HindIII*, respectively, and annealed with probe to cloned ARV-2 DNA. Lanes i, j, DNA from HUT-78 cells infected with ARV-4, digested with *SacI* and *PstI*, respectively and annealed with probe to cloned ARV-2 DNA.

LAV and HTLV-III are related (our unpublished data; M. Bryant and M. Gardner, personal communication). DNA sequence analysis of these cloned ARV DNA molecules will reveal the genetic content of the virus and will provide a basis for comparisons with other viruses. Preliminary sequencing of ARV has shown small amounts of homology in portions of the gag and pol proteins of many retroviruses including Rous sarcoma virus, squirrel-monkey retrovirus and HTLV-I (our unpublished data). The significance of polymorphism of nucleotide sequences in independent isolates remains to be evaluated.

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The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus

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Acquired immune deficiency syndrome (AIDS) is characterized by opportunistic infections and by 'opportunistic neoplasms' (for example, Kaposi's sarcoma)¹. Persistent generalized lymphadenopathy (PGL) is epidemiologically associated with AIDS, especially in male homosexuals. A subset of T lymphocytes positive for the CD4 antigen² (also termed T4 antigen), is depleted in AIDS and PGL patients. A retrovirus found in T-cell cultures from these patients³⁻⁵ is strongly implicated in the aetiology of AIDS because of the high frequency of isolation⁴ and the prevalence of specific antibodies⁶⁻⁸ in the patients. Here we have detected cell-surface receptors for the AIDS retrovirus (human T-cell leukaemia virus-III (HTLV-III) and lymphadenopathy-associated virus-1 (LAV-1) isolates) by testing the susceptibility of cells to infection with pseudotypes of vesicular stomatitis virus bearing retroviral envelope antigens, and by the formation of multinucleated syncytia on mixing virus-producing cells with receptor-bearing cells. Receptors were present only on cells expressing CD4 antigen; among 155 monoclonal antibodies tested, each of the 14 anti-CD4 antibodies inhibited formation of syncytia and blocked pseudotypes. Productive infection of CD4⁺ cells with HTLV-III or LAV-1 markedly reduced cell-surface expression of CD4. In contrast, receptors for HTLV-I and HTLV-II were not restricted to CD4⁺ cells, were not blocked by anti-CD4 antibodies; cells productively infected with HTLV-I and HTLV-II expressed surface CD4. Hence, we conclude that the CD4 antigen is an essential and specific component of the receptor for the causative agent of AIDS.

The first reported isolate of the AIDS retrovirus³ was named lymphadenopathy-associated virus as it was made from a PGL patient; two further isolates from AIDS patients in France were named immune deficiency-associated virus (IDAV-1, IDAV-2)^{9,10}, and American isolates⁴ have been collectively named HTLV-III. As the core proteins of the AIDS retroviruses isolated in France and America are closely related antigenically (M. G. Sarngadharan, R. C. Gallo and L. Montagnier, personal communication) and antisera from British AIDS and PGL patients specific for viral membrane antigens are cross-absorbed by virus from either source⁸, we consider LAV, IDAV and HTLV-III to be a single species of virus. Given the distant relationship of the AIDS virus to HTLV-I and HTLV-II (refs 11, 12) and as the term 'human T-lymphotropic virus' was originally coined for this family of retroviruses by Barré-Sinoussi *et al.*³, we shall use the general name, HTLV-III, for the AIDS retrovirus. The particular isolates used in the present study were LAV-1 (ref. 3) and HTLV-IIIIB (ref. 4).

HTLV-I, HTLV-II and HTLV-III each show a tropism for CD4⁺ T cells¹³⁻¹⁵ although some non-T cells can be infected *in vitro*¹⁶⁻¹⁸. We have sought to characterize the cell-surface receptors for these viruses and to ascertain whether the tropism of the viruses is determined at the receptor level. As there are no quantitative infectivity assays for HTLV, we identified receptor-positive cells by assessing induction of multinucleated syncytia^{18,19}, and by the susceptibility of various cell types to pseudotypes of vesicular stomatitis virus (VSV) bearing envelope

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1. Barré-Sinoussi, F. *et al.* *Science* **220**, 868-871 (1983).
2. Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. *Science* **224**, 497-500 (1984).
3. Gallo, R. C. *et al.* *Science* **224**, 500-503 (1984).
4. Levy, J. A. *et al.* *Science* **225**, 840-842 (1984).
5. Gazdar, A. F. *et al.* *Blood* **55**, 409-416 (1980).
6. Bailey, J. M. & Davidson, N. *Analyt. Biochem.* **70**, 75-85 (1976).
7. Shank, P. R. *et al.* *Cell* **15**, 1383-1396 (1978).
8. Southern, E. M. *J. molec. Biol.* **98**, 503 (1975).
9. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205 (1980).
10. Luciw, P. A., Oppermann, H., Bishop, J. M. & Varmus, H. E. *Molec. cell. Biol.* **4**, 1260-1269 (1984).
11. Karn, J., Brenner, S. & Barnett, L. *Meth. Enzym.* **101**, 3-19 (1983).
12. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning, a Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).

Table 1 Receptors for HTLV subtypes

Cells		VSV pseudotypes of			
Type	Line	HTLV-I	HTLV-II	HTLV-III	LAV-1
Human T cell	JM	+	+	+	+
Human T cell	CEM	+	+	+	+
Human T cell	CEM/LAV-1	+	+	+	+
Human sarcoma	HOS	+	+	—	—
Human sarcoma	HOS/HTLV-I	—	—	—	NT
Human carcinoma	HeLa	+	+	—	—
Cat kidney	CCC	+	+	—	—
Cat kidney	CCC/HTLV-I	—	—	—	NT

Pseudotypes of VSV (Indiana strain, wild type) were prepared as described previously²⁰ by adding excess neutralizing sheep anti-VSV serum to VSV collected from cells pre-infected with the HTLV strains. The pseudotype titres ranged between 10^4 and 10^5 PFU ml⁻¹. VSV(HTLV) pseudotypes were plated on the indicator cells in the presence of $2 \mu\text{g ml}^{-1}$ DEAE-dextran at $10^{3.5}$, $10^{2.5}$ and $10^{1.5}$ PFU per well. HOS, HeLa and CCC cells were infected as monolayers (10^6 cells per 30 mm-diameter well), whereas JM and CEM cells were infected in suspension. Monolayer cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS), and suspension cells were grown in RPMI 1640 + 10% FCS. CEM/LAV-1 were CEM cells productively infected with LAV-1 (ref. 8); HOS/HTLV-I and CCC/HTLV-I were productively infected with HTLV-I (refs 17, 20). After virus adsorption, the cells were washed and monolayer cells were overlaid with agar medium; suspension cells were plated in a layer of agar medium above a monolayer of 10^6 bovine MDBK cells (not susceptible to any strain of HTLV) to act as indicator cells for progeny VSV released from infected cells in suspension. Plaques were counted 2 or 3 days after plating of the pseudotypes. + Indicates 10^3 PFU ml⁻¹; — indicates 10 PFU ml⁻¹ for the cell types assayed; NT, not tested.

antigens of the different HTLV strains²⁰. When cells infected with retroviruses are superinfected with VSV, a proportion of the progeny VSV assemble sufficient envelope glycoprotein of the retrovirus to resist neutralization by hyperimmune anti-VSV serum²¹. The host range of such 'pseudotype' virions is restricted to cells expressing receptors specific to the retrovirus^{22,23}. Following penetration of the cell and uncoating of the virion, however, the transcribed VSV genome replicates to produce nonpseudotype particles which infect and destroy neighbouring cells. Thus, VSV(HTLV) pseudotypes provide a simple cytopathic plaque assay for HTLV receptors²⁰.

VSV pseudotypes were prepared with the envelopes of each HTLV species and their ability to form plaques was tested on different cells. Table 1 shows that VSV(HTLV-I) and VSV(HTLV-II) plate on a variety of cell types, whereas VSV(HTLV-III) is restricted to the two T-cell lines tested. CEM cells pre-infected with LAV-1 blocked pseudotypes of both LAV-1 and HTLV-IIIB (the French and American isolates) but

not pseudotypes of HTLV-I and HTLV-II. HOS and CCC cells pre-infected with HTLV-I blocked pseudotypes of both HTLV-I and HTLV-II. These data indicate that HTLV-I and HTLV-II utilize the same receptor which is expressed on lymphoid, fibroblastic and epitheloid cell types, whereas HTLV-III uses a different receptor with a more restricted distribution.

We have shown previously^{19,20} that extensive cell fusion occurs when uninfected cells bearing HTLV receptors are mixed with cells producing HTLV-I or HTLV-II. As cells infected with HTLV-III have been observed to form multinucleated giant cells^{5,10}, we tested whether syncytia would be induced on mixing HTLV-III-producing HT/H9 cells⁵ with a 10-fold excess of uninfected CEM, JM or HT/H4 indicator cells. Many syncytia were observed with each cell type, commencing as early as 6 h after mixing and increasing in size and number until 48 h, when up to 50% of nuclei were contained within syncytia. No syncytia appeared after mixing HTLV-III-producing cells with HOS, HeLa or CCC cells, although these indicator cells formed abundant syncytia with HTLV-I and HTLV-II. Thus, syncytium induction can be used as an alternative to the pseudotype method for determining the presence of HTLV-III receptors on the surface of uninfected cells.

In a more extensive set of human cell lines, we used both syncytium induction and pseudotype infection to determine the presence of HTLV-III receptors; at the same time we determined the presence of the CD4 and CD5 antigens using monoclonal antibodies (CD5 is a 'pan-T' marker also called T1). Table 2 shows that only the cells expressing CD4 antigen were sensitive to syncytium induction by HTLV-III and infection by VSV(HTLV-III) pseudotypes. In particular, two non-T-cell lines, U937 and LCo-B, which expressed CD4 antigen but not the pan-T marker, expressed HTLV-III receptors. Thus, the CD4 antigen may be a component of the HTLV-III receptor.

Antibodies to antigens situated in or adjacent to the receptor site for HTLV-III might be expected to compete in binding to or obscuring the receptor, as has been shown for baboon endogenous virus²⁴. We therefore screened 155 monoclonal antibodies to T-cell surface antigens for their ability to inhibit the formation of syncytia induced by HTLV-III; these included 135 monoclonal antibodies from the 2nd International Workshop on Human Leukocyte Differentiation Antigens, which were screened without knowledge of their antigen specificity. Table 3 shows that all the monoclonal antibodies to CD4 antigen blocked cell fusion induced by HTLV-III. In addition, five other monoclonal antibodies (Leu8, two anti-MHC class II monoclonal antibodies and W80 and W151, for which the antigens

Table 2 Correlation of HTLV-III receptors with CD4 antigen expression

Indicator cells		HTLV-III receptor tests		% Cells immunofluorescent	
Origin	Designation	Syncytium induction	Pseudotype infection	Anti-CD4 (MT310)	Anti-CD5 (UCHT2)
T-cell leukaemia	JM	+++	+	100	100
T-cell leukaemia	CEM	+++	+	100	100
T-cell leukaemia	HT/H4	+++	NT	100	100
T-cell leukaemia	HT/H9	++	NT	90	90
T-cell leukaemia	MOLT-4	+++	+	100	90
T-cell leukaemia	HSB2	—	—	0	80
T-cell leukaemia	GH1	+	+	80	90
T-cell lymphoma (Sezary)	HUT 78	++	NT	30	70
HTLV-I-transformed T cell	C10/MJ2	++	+	90	90
HTLV-II-transformed T cell	Ton-1	+	+	90	100
Burkitt's lymphoma (B cell)	Raji	—	—	0	0
B-lymphoblastoid (EBV)	LCo-B	+	—	30	0
Nine other B-lymphoblastoid lines	—	—	NT	0	0
Promyelocytic leukaemia	HL60	—	NT	0	0
Monocytic leukaemia	U937	++	+	70	0
Osteogenic sarcoma	HOS	—	—	0	0
Cervical carcinoma	HeLa	—	—	0	0

Twenty-five established human cell lines (listed above) were tested for expression of CD4 and CD5 antigens, for syncytial response to HTLV-III and for susceptibility to infection by VSV(HTLV-IIIB) and VSV(LAV-1) pseudotypes.

have not been classified by the Workshop) weakly but consistently inhibited syncytium formation. To determine whether the antibodies were binding to indicator cells or to virus-producing cells, four anti-CD4 antibodies with syncytium-inhibiting properties were incubated separately with receptor-bearing cells and virus-producing cells, and the cells were mixed together after washing three times in antibody-free medium. In each case, strong inhibition of cell fusion was observed when antibody was preadsorbed to indicator cells but not when antibody was preadsorbed to virus-producing cells, indicating that the antibodies blocked syncytium formation by binding to cell receptor sites rather than to viral envelope antigens.

Monoclonal antibodies that were positive for syncytium inhibition, and an equivalent number of negative monoclonal antibodies selected from the different antigen groups, were tested for their ability to block VSV(HTLV-III) infection of CEM or GH1 cells, by incubating the cells with antibody before plating the pseudotype. Table 3 shows that all the anti-CD4 monoclonal antibodies, but not Leu8, W80 or W151, caused significant reductions (>80%) of pseudotype plaque-forming units (PFU). With some monoclonal antibodies, however, pseudotype inhibition was not always achieved, even at high concentrations; each

Table 3 Blocking of HTLV-III receptors by monoclonal antibodies

Antibody		No. of monoclonal antibodies positive/tested	
Group designation	Specificity	Syncytium inhibition	Pseudotype inhibition
CD1	Cortical thymocyte	0/9	NT
CD2	Pan-T, E receptor (T11)	0/16	0/1
CD3	Pan-T	0/13	0/2
CD5	Pan-T+B-CLL (T1)	0/11	0/4
CD6	Pan-T	0/7	NT
CD7	Pan-T	0/11	NT
CD4	Helper/inducer (T4)	14/14	14/14
CD8	Suppressor/cytotoxic (T8)	0/21	0/2
CD25	Interleukin-2 receptor (Tac)	0/9	0/1
CD26	Activation antigen p120	0/4	NT
—	Unclassified workshop	2/16	0/3
—	monoclonal antibodies		
—	Workshop positive and negative controls	0/11	0/3
—	T subset (Leu8)	1/1	0/1
—	T subset (S2)	0/1	0/1
—	MHC class I	0/1	0/1
—	MHC class II	2/8	0/5
—	β_2 -microglobulin	0/2	0/1

We tested 135 monoclonal antibodies supplied for the 2nd International Workshop on Human Leukocyte Differentiation Antigens for their ability to inhibit syncytium formation induced by HTLV-III_B; 20 further monoclonal antibodies were also tested. Two indicator cell lines, JM and HT/H4, tested separately for syncytium inhibition by each monoclonal antibody, both gave the same results. JM cells were positive for all the known CD antigen groups tested. Monoclonal antibodies were used at 1:20 dilutions in each test, and appearance of syncytia was read at 6 and 24 h after mixing HTLV-III-producing cells with indicator cells and monoclonal antibodies. In the first two experiments, all the monoclonal antibodies were coded and syncytia were independently scored by two authors, neither of whom knew the antigen specificity of the antibodies. Cultures showing >80% reduction of numbers of syncytia at 24 h were scored as positive. All the anti-CD4 monoclonal antibodies and 25 other monoclonal antibodies were also tested for pseudotype inhibition by treating indicator cells with $\sim 80 \mu\text{g ml}^{-1}$ antibody before adding VSV(HTLV-III_B) or VSV(LAV-1). For the pseudotype assays, 5×10^5 cells per 30-mm well of an adherent subline of CEM cells (and, in one experiment, adherent GH1 cells) were used as indicator cells. After virus adsorption, 10^6 HOS cells were added to the adherent T cells to form a dense layer for the enhancement of VSV plaque formation. Monoclonal antibodies giving >80% decrease in numbers of PFU were scored as inhibitory. Two or more independent experiments were done for each monoclonal antibody tested. The anti-CD4 antibodies were W68, W69, W70, W71, W75, W77, W78, W79, W107, W113, MT151, MT130, MT321 and Leu3a.

was tested in at least three separate experiments. Most monoclonal antibodies were tested at a 1:20 dilution, representing ~ 50 – $100 \mu\text{g}$ immunoglobulin per ml of medium. Four inhibitory anti-CD4 monoclonal antibodies with known immunoglobulin concentrations were titrated to determine the highest five-fold dilution inhibiting $\geq 80\%$ of VSV(HTLV-III) PFU. Two monoclonal antibodies (MT310 and MT321) had end-point titres of $16 \mu\text{g}$ immunoglobulin per ml, whereas Leu3a was positive at $3.2 \mu\text{g}$ immunoglobulin per ml and MT151 at $0.64 \mu\text{g}$ immunoglobulin per ml. The same monoclonal antibodies failed to inhibit the plating of VSV(HTLV-I) pseudotypes on CEM cells even at $100 \mu\text{g}$ immunoglobulin per ml.

The results of the blind screen of the antibodies from the 2nd International Workshop and the more extensive testing of selected monoclonal antibodies (Table 3) show that antibodies to CD4 antigen specifically prevent the HTLV-III envelope antigens from interacting functionally with the cell surface, presumably by binding to sites coincident with or adjacent to virus receptors. The significance of the three other monoclonal antibodies that partially blocked HTLV-III receptors is not yet clear.

Our experiments demonstrate that either pre-incubation with monoclonal antibodies to CD4 (Table 3) or pre-infection with the LAV-1 isolate of HTLV-III (Table 1) blocks the reception or penetration of VSV(HTLV-III) pseudotypes in CEM cells. We therefore tested whether productive infection of CD4-positive cells with HTLV-III alters the expression of the CD4 antigen at the cell surface. Table 4 summarizes the results of immunofluorescence assays performed on uninfected and HTLV-infected cells for CD4 antigen and for HTLV membrane antigens. We found a reciprocal relationship for expression of CD4 and HTLV-III antigens on the cell surface. In contrast, cells expressing membrane antigens of HTLV-I and HTLV-II also expressed CD4 antigen at a high level. Figure 1 shows the quantitation of anti-CD4 immunofluorescence by flow cytometry of three cell lines before and after HTLV-III infection. These results indicated that the amount of CD4 antigen available at the cell surface for monoclonal antibody binding was greatly reduced in HTLV-III-infected cells, whereas expression of major histocompatibility complex (MHC) class I (Fig. 1) and other membrane antigens (CD2, CD3 and CD25, data not shown) was unaffected.

Our results show that HTLV-III receptors are restricted to cells expressing CD4 antigen, suggesting that the CD4 antigen

Table 4 Reciprocal expression of HTLV and CD4 antigens on the cell surface

Lymphoid cell lines		Immunofluorescence	
Virus	Line	Anti-CD4	Anti-HTLV-III
Uninfected	H9	+++	—
	CEM	+++	—
	LCo-B	+	—
HTLV-III	H9/HTLV-III	±	+++
	H9/LAV-1	±	+++
	CEM/LAV-1	±	+++
	LCo-B/LAV-1	±	++
HTLV-I	C91/PL	+++	Anti-HTLV-I +++
	C10/MJ2	+++	++
HTLV-II	C12/18M	+++	Anti-HTLV-II +++
	Ton-1	+++	++

The cells were washed and treated without fixation with monoclonal MT151 (anti-CD4) followed by fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse IgG (Miles), or with sera from human patients specific for HTLV-I, HTLV-II or HTLV-III, followed by FITC-labelled goat anti-human IgG (Miles). Control human sera and control mouse immunoglobulin gave insignificant fluorescence. The results were scored by eye using a fluorescence microscope. 'Plus' marks indicate the intensity of fluorescence rather than the proportion of cells fluorescing.

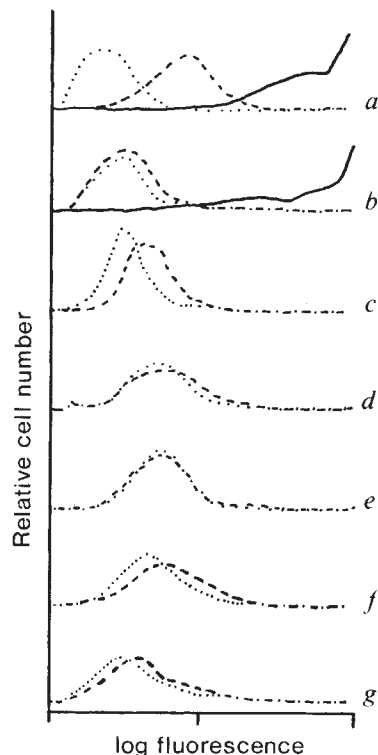


Fig. 1 Fluorescence histograms showing binding of monoclonal antibodies to uninfected and virus-infected cells. *a*, CEM; *b*, CEM/LAV-1; *c*, H9; *d*, H9/HTLV-III; *e*, H9/LAV-1; *f*, LCo-B; *g*, LCo-B/LAV-1. Solid lines, anti-MHC class I antibody; broken lines, anti-T4 antibody; dotted lines, control FITC-conjugated antibody. For fluorescence staining, 5×10^5 cells were incubated for 30 min on ice with 100 μ l of monoclonal antibody, washed once with 2 ml of cold medium (RPMI 1640 with 3% FCS and 0.02% sodium azide) and incubated for a further 30 min with 100 μ l of a saturating concentration of FITC-conjugated goat anti-mouse immunoglobulin (Sigma). After a further wash, the cells were fixed overnight in 0.5 ml of 3% formalin in phosphate-buffered saline with 1% FCS; 10,000 cells were analysed on a fluorescence-activated cell sorter fitted with a log amplifier. The anti-MHC class I antibody was W6/32; the anti-CD4 antibody was Leu3a. Controls comprised cells treated with FITC-labelled goat anti-mouse immunoglobulin alone.

itself is a necessary component of the receptor. Receptor blocking by monoclonal antibodies required high concentrations of antibody which were more effective in inhibiting formation of syncytia than in preventing infection by pseudotypes. Cell fusion induced by HTLV-III may require many more receptors than those required for the endocytosis of VSV(HTLV-III) particles, which may explain the relatively greater sensitivity to antibody of the syncytium inhibition assay.

In the accompanying paper, Klatzmann *et al.*²⁵ show that incubation of CD4⁺ cells in the presence of anti-CD4 antibodies, for the first 2 weeks of infection, inhibits the replication of HTLV-III (LAV-1), indicating that the CD4 antigen is required at some stage of the HTLV-III replication cycle. The diminution of CD4 expression on the surface of HTLV-III-infected cells (Table 4, Fig. 1) has also been observed by other investigators (refs 15, 25 and M. Popovic, personal communication); this finding may result simply from the viral envelope antigen competitively masking the binding sites for the monoclonal antibodies. Alternatively, the formation of a complex between the viral envelope glycoprotein and the CD4 receptor may result in a block to maturation, or rapid internalization, or capping and shedding of the CD4 antigen.

The CD4 antigen is a single-chain polypeptide of relative molecular mass 62,000², found on a T-cell subset which includes helper cells (for B-cell and macrophage responses), T cells which activate T suppressor cells and some cytotoxic T cells^{26,27}. There is evidence that when CD4⁺ T cells recognize antigen presented

by accessory cells, CD4 may interact with MHC class II antigens (for example, HLA-DR) expressed on the antigen-presenting cell^{28,29}. It is conceivable that the recognition of CD4 by the HTLV-III envelope antigen involves molecular mimicry of class II antigens; our preliminary data indicate that two out of eight monoclonal antibodies to class II antigens suppress formation of HTLV-III syncytia. Three other monoclonal antibodies (W80, W151 and Leu8) that are apparently not specific to CD4 antigen also blocked HTLV-III syncytia, but were less effective in blocking pseudotypes; these antibodies may recognize antigen-bearing molecules other than CD4 that also participate in HTLV-III reception and penetration, they may sterically hinder the presentation of CD4 to HTLV-III envelope antigen, or they may be required at a later stage in the process of cell fusion. It is noteworthy that the Leu8 antigen²⁶ is a marker for that subset of CD4⁺ cells that shows greatest depletion in AIDS and PGL³⁰.

The expression of CD4 antigen is not restricted entirely to subsets of T cells. Among the cells we studied (Table 2), the LCo-B lymphoblastoid line transformed by Epstein-Barr virus (EBV) is unusual in expressing CD4; nine other B-lymphoblastoid lines were negative for CD4 antigen and for HTLV-III receptors. The U937 monocytic cell line derived from a histiocytic lymphoma³¹ has been reported previously to express CD4 antigen as well as some normal cells of the monocyte-macrophage lineage^{32,33}. This raises the question of whether monocytes and macrophages may become infected by HTLV-III *in vivo*, as macrophage function is severely compromised in AIDS¹.

Most naturally-occurring, infectious transmitted retroviruses, such as avian, feline and bovine leukaemia viruses, use receptors common to many cell types²³, as shown here (Table 1) for HTLV-I and HTLV-II. In contrast, HTLV-III recognizes a differentiation antigen. Some other viruses also appear to use receptors specific to the cell type most affected by infection, for example, the complement receptor type 2 for EBV³⁴, the acetylcholine receptor for rabies virus³⁵ and the β -adrenergic receptor for reovirus (B. Fields and M. Green, personal communication). AIDS and PGL are syndromes resulting primarily from a depletion of CD4⁺ Leu8⁺ cells in the peripheral blood and lymph nodes^{1,30}. Our finding that the CD4 antigen itself is an essential component of the receptor for the aetiological virus of AIDS helps to explain the nature of the disease. It remains to be seen whether receptor restriction is the sole determinant of cell tropism for HTLV-III.

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1. Pinching, A. J. *Clin. exp. Immun.* **56**, 1-13 (1984).
2. Terhost, C., van Agthoven, A., Reinherz, E. & Schlossman, S. *Science* **209**, 520-521 (1980).
3. Barré-Sinoussi, F. *et al. Science* **220**, 868-870 (1983).
4. Gallo, R. C. *et al. Science* **224**, 500-503 (1984).
5. Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. *Science* **224**, 497-500 (1984).
6. Brun-Vezinet, F. *et al. Lancet* **i**, 1253-1256 (1984).
7. Safai, B. *et al. Lancet* **i**, 1438-1440 (1984).
8. Cheingsong-Popov, R. *et al. Lancet* **ii**, 477-480 (1984).
9. Vilmer, E. *et al. Lancet* **i**, 753-757 (1984).
10. Montagnier, L. *et al. in Human T-cell Leukemia/Lymphoma Virus* (eds Gallo, R. C., Essex, M. & Gross, L.) 363-379 (Cold Spring Harbor Laboratory, New York, 1984).
11. Schüpbach, J. *et al. Science* **224**, 503-506 (1984).
12. Arya, S. K. *et al. Science* **225**, 927-980 (1984).
13. Popovic, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 5402-5406 (1983).
14. Chen, I. S. Y., Quan, S. G. & Golde, D. W. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7006-7009 (1983).
15. Klatzmann, D. *et al. Science* **225**, 59-63 (1984).
16. Montagnier, L. *et al. Science* **225**, 63-66 (1984).
17. Clapham, P., Nagy, K., Cheingsong-Popov, R. & Weiss, R. A. *Science* **222**, 1125-1127 (1983).
18. Hoshino, H., Shimoyama, M., Miwa, M. & Sugimura, T. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7337-7341 (1983).
19. Nagy, K., Clapham, P., Cheingsong-Popov, R. & Weiss, R. A. *Int. J. Cancer* **32**, 321-328 (1983).
20. Clapham, P., Nagy, K. & Weiss, R. A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2886-2889 (1984).
21. Zavada, J. *J. gen. Virol.* **15**, 183-191 (1972).

22. Boettiger, D., Love, D. N. & Weiss, R. A. *J. Virol.* **15**, 108-114 (1975).
23. Weiss, R. A. in *Virus Receptors* (eds Lonberg-Holm, K. & Philipson, L.) 186-202 (Chapman & Hall, London, 1981).
24. Thiry, L. *et al. J. Virol.* **48**, 697-708 (1983).
25. Klatzmann, D. *et al. Nature* **312**, 767-771 (1984).
26. Gatenby, P. A. *et al. J. Immun.* **129**, 1997-2000 (1982).
27. Bernard, A. *et al. (eds) Leucocyte Typing* (Springer, Berlin, 1984).
28. Meur, S. C., Schlossman, S. F. & Reinherz, E. L. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4395-4399 (1982).
29. Biddison, W. E. *et al. J. exp. Med.* **159**, 783-797 (1984).
30. Nicholson, J. K. A. *et al. J. clin. Invest.* **73**, 191-201 (1984).
31. Sundström, C. & Nilsson, K. *Int. J. Cancer* **17**, 565-577 (1976).
32. Wood, G. S., Warner, N. L. & Warnke, R. A. *J. Immun.* **131**, 212-216 (1983).
33. Moscicki, R. A. *et al. J. Immun.* **131**, 743-748 (1983).
34. Fingerhuth, J. D. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 4510-4514 (1984).
35. Lentz, T. L. *et al. Science* **215**, 182-184 (1982).

T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV

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Many viruses, including retroviruses, are characterized by their specific cell tropism¹⁻³. Lymphadenopathy-associated virus (LAV) is a human lymphotropic retrovirus isolated from patients with acquired immune deficiency syndrome (AIDS) or related syndromes^{4,5}, that displays selective tropism for a subset of T lymphocytes defined by the expression of a surface glycoprotein of relative molecular mass 62,000 (62K) termed T4 (refs 6-8). This glycoprotein delineates a subset of T lymphocytes with mainly helper/inducer functions, while T lymphocytes of the reciprocal subset express a glycoprotein termed T8, have mainly cytotoxic/suppressor activities, and are unable to replicate LAV^{7,9}. Such a tropism may be controlled at the genomic level by regulatory sequences, as described for the human T-cell leukaemia viruses HTLV-I and -II (refs 2, 3). Alternatively or concomitantly, productive cell infection may be controlled at the membrane level, requiring the interaction of a specific cellular receptor with the virus envelope, as demonstrated recently for Epstein-Barr virus (EBV)¹. Therefore, we have investigated whether the T4 molecule itself is related to the receptor for LAV. We report here that preincubation of T4⁺ lymphocytes with three individual monoclonal antibodies directed at the T4 glycoprotein blocked cell infection by LAV. This blocking effect was specific, as other monoclonal antibodies—such as antibody to histocompatibility locus antigen (HLA) class II or anti-T-cell natural killer (TNK) target—directed at other surface structures strongly expressed on activated cultured T4⁺ cells, did not prevent LAV infection. Direct virus neutralization

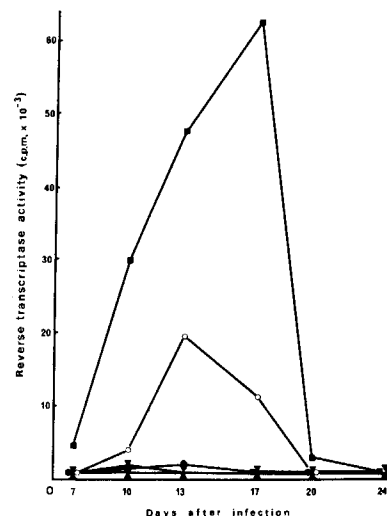


Fig. 1 Monoclonal anti-T4 antibodies, but not anti-HLA class II or anti-TNK-target, inhibit replication of LAV in T4⁺ cultured cells. ■, Anti-HLA class II monoclonal antibodies (mixture of CA2.06, CA1.41 and BM50); ○, anti-TNK-target antibody; ●, 12T4 anti-T4 antibody; ▼, OKT4 anti-T4 antibody; ▲, 19Thy anti-T4 antibody. Cell cultures and determinations of reverse transcriptase activity were performed as described in Table 1.

by monoclonal antibodies was also ruled out. These results strongly support the view that a surface molecule directly involved in cellular functions acts as, or is related to, the receptor for a human retrovirus.

While studying the LAV selective tropism, we observed that, at the time of virus replication, a decreased proportion of purified cultured LAV-infected T4⁺ lymphocytes was labelled by immunofluorescence staining with anti-T4 monoclonal antibodies (Table 1)⁷. As at that time the remaining viable cells retained their specific T-cell marker T3 (ref. 9), we assumed that this phenomenon could not be related to the cytopathic effect of LAV⁷, but was probably due to either modulation of T4 molecules at the cell membrane or steric hindrance of antibody-binding sites. Both cases imply interaction of viral particles produced in the culture with the expression of T4. Consequently, we designed experiments to assess whether the T4⁺ tropism of LAV was directly related to the presence of this glycoprotein on T4⁺ lymphocytes.

Three antibodies (OKT4, Coulter clone 12T4D11 and DFCI clone 19 Thy 5D7) specific for distinct epitopes of the T4 glycoprotein were used in these studies. As controls we used four different monoclonal antibodies, three directed against HLA class II gene products, mainly the DR molecule (given by D. Charron)¹⁰, and anti-TNK-target, which recognizes a 140K surface molecule strongly expressed on all cultured T lymphocytes¹¹. T4⁺ lymphocytes (stimulated with phytohaemagglutinin (PHA) for 3 days) were obtained from peripheral blood mononuclear cells of normal donors as described previously^{6,7},

Table 1 Reduced expression of T4 molecule in LAV-infected T4⁺ lymphocytes

Cell fraction	LAV	% Lymphocytes stained					
		Before the peak of RT (day 4)			After the peak of RT (day 11)		
		OKT4	OKT4A	OKT8	OKT4	OKT4A	OKT8
T4 ⁺	—	95	94	3	78	76	1
T4 ⁺	+	85	89	1	21	25	3
T8 ⁺	—	1	NT	95	NT	NT	94
T8 ⁺	+	1	NT	85	NT	NT	87

Purified T4⁺ and T8⁺ lymphocytes were obtained by cellular affinity chromatography as described previously^{6,7} and cultured in the presence of 1% PHA-M in RPMI1640 medium supplemented with 10% fetal calf serum. After 3 days, 5×10^6 cells were incubated for 30 min at 37 °C with 50,000 c.p.m. of LAV reverse transcriptase-containing supernatant. After washing, cells were resuspended in 5 ml of culture medium containing IL-2, anti- γ -interferon serum and polybrene⁴. Reverse transcriptase activity (RT) was determined twice weekly in cell-free supernatants as reported elsewhere⁴. In this experiment, RT peaked at day 8, having reached 1×10^5 c.p.m. Indirect immunofluorescence staining was performed as described previously using the OKT4, OKT4A and OKT8 monoclonal antibodies (Ortho-Diagnostics System)⁶.

Table 2 Monoclonal antibodies inhibit infection of lymphocytes by LAV

Antibody	Reverse transcriptase activity (c.p.m. $\times 10^{-3}$)	Lymphocyte concentration ($\times 10^6 \text{ ml}^{-1}$)
-	39	1.3
Anti-T4	2	1.2
Anti-class II	54	1.4

Purified T4⁺ lymphocytes were obtained and cultured as described for Table 1. After 3 days, cells were centrifuged and 5×10^6 cells incubated with 500 μl of culture medium containing saturating concentrations (1:200) of the respective antibodies or medium alone as control. After 20 min at room temperature, the cells were washed once and incubated for 30 min at 37 °C with 50,000 c.p.m. of LAV reverse transcriptase-containing supernatant. After washing, cells were resuspended in 5 ml of culture medium to which the respective monoclonal antibody had been added at a 1:400 dilution. Reverse transcriptase activity was determined twice weekly for cell-free supernatants obtained at the time when cultures were adjusted to 1×10^6 cells per ml and new medium added⁴. The results shown were obtained at the peak of virus production in this experiment (day 6 after infection). Lymphocyte concentrations represent cell growth from day 3 (when the number of cells was adjusted to $1 \times 10^6 \text{ ml}^{-1}$) to day 6.

Table 3 Incubation with monoclonal antibodies does not neutralize LAV

LAV	Antibody	Reverse transcriptase activity (c.p.m. $\times 10^{-3}$)
-	-	1
+	-	32
+	T3	35
+	T4	31
+	T8	30

LAV-containing supernatant was incubated for 30 min at 37 °C with either OKT3, OKT4 or OKT8 (Ortho-Diagnostic) monoclonal antibody at the concentrations used in the previous experiments; 2 ml of this supernatant were then mixed with 1 ml of goat anti-mouse immunoglobulin-coated Sepharose 6-MB beads. After incubation of this mixture for 30 min at room temperature, reverse transcriptase activity was measured in 2 ml of the supernatant.

and incubated for 20 min at room temperature with saturating concentrations of antibodies. After washing, cells were incubated with LAV-containing supernatant for 30 min at 37 °C, using 10,000 c.p.m. of reverse transcriptase activity per 10^6 cells⁴. Unadsorbed virus was then washed out and cells were cultured in medium containing anti-interferon serum, interleukin-2 (IL-2)⁴ and the respective monoclonal antibodies. As infectious particles can persist in culture medium even after washing and further infect lymphocytes that are no longer coated by antibodies shed in the medium, we added antibodies to the culture for the first 2 weeks to prevent such late infection.

Incubation and culture of T4⁺ cells with anti-TNK-target or anti-HLA class II monoclonal antibodies did not prevent virus production, as detected by reverse transcriptase activity in the culture supernatants as well as by the appearance of a cytopathic effect⁷. In contrast, incubation with either of the anti-T4 antibodies completely inhibited virus replication in these cells (Table 2, Fig. 1); we could detect no reverse transcriptase activity or cytopathic effect for as long as 1 month after virus infection, even after discontinuing the addition of anti-T4 antibody to culture medium on day 13 (Fig. 1). Such results could not be attributed to the decreased proliferation of cultured cells in the presence of these monoclonal antibodies (Table 1; ref. 12). Neutralization of LAV particles by anti-T4 antibody was ruled out. Viruses were not retained by the goat anti-mouse immunoglobulin-coated Sepharose 6-MB beads which were used to enrich the T4⁺ population^{6,7} after incubation with these

antibodies, at the ratios and concentrations used in the previous experiments (Table 3). Nonspecific blockade of virus penetration by modification of the membrane properties after antibody binding was also unlikely, as other monoclonal antibodies which bind to glycoproteins expressed in high amounts on the membranes of activated cells did not prevent viral infection.

In the conditions used, the amount of reverse transcriptase activity appeared to vary according to the antibody used. The possibility that some of these antibodies could even enhance virus penetration or production (Table 2) is being investigated.

Thus, these results strongly suggest that LAV tropism depends on the interaction of LAV with the T4 molecule; they are in accord with the data presented in the accompanying paper¹³, which show the relationship between the T4 expression of different lymphoid cell lines and their susceptibility to LAV and HTLV-III, a similar retrovirus isolated from AIDS patients¹⁴. Replication of LAV in EBV-transformed lymphoblastoid cell lines¹⁵ could indeed be associated with a significant T4 expression on these cells¹³.

The present findings strongly suggest that the T4 glycoprotein is at least associated with all or part of the receptor for LAV, of particular interest in view of the involvement of this molecule in human T-cell functions. Indeed, this glycoprotein delineates a subset of lymphocytes which primarily mediate induction of the immune response^{8,9}. Furthermore, recent studies have suggested that T4 is involved in the recognition of HLA class II gene products^{16,17}. Taken together, these data provide a molecular basis for the previously described tropism of LAV for the inducer lymphocyte subset. In addition, they may more generally reflect one of the mechanisms of interaction between retroviruses and immunocompetent cells. Finally, these results should help to delineate future therapeutic strategies in the treatment of AIDS.

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1. Fingerhuth, J. D. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 4510-4514 (1984).
2. Leuz, J. *et al. Nature* **308**, 467-470 (1984).
3. Chen, I. S., McLaughlin, J. & Golden, D. W. *Nature* **309**, 276-279 (1984).
4. Barre Sinoussi, F. *et al. Science* **220**, 868-871 (1983).
5. Vilmer, E. *et al. Lancet* **i**, 753-756 (1984).
6. Klatzmann, D. *et al. Ann. N.Y. Acad. Sci.* (in the press).
7. Klatzmann, D. *et al. Science* **225**, 59-63 (1984).
8. Reinherz, E. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 4061-4065 (1979).
9. Reinherz, E. L. & Schlossman, S. F. *Cell* **19**, 821-827 (1980).
10. Charron, D. J. & McDewitt, M. O. *J. exp. Med.* **152**, 18s-36s (1980).
11. Hercend, T. *Eur. J. Immun.* **14**, 844-852 (1984).
12. Engleman, E. G. *et al. J. Immun.* **130**, 2623-2628 (1983).
13. Dalgleish, A. *et al. Nature* **312**, 763-767 (1984).
14. Gallo, R. C. *et al. Science* **224**, 500-504 (1984).
15. Montagnier, L. *et al. Science* **225**, 63-66 (1984).
16. Meuer, S. C., Schlossman, S. F. & Reinherz, E. L. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4395-4399 (1982).
17. Biddison, W. E. *et al. J. exp. Med.* **159**, 783-797 (1984).

Cloning, sequence and expression of human interleukin-2 receptor

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T lymphocytes, essential for the generation of a normal immune response, require the presence of the lymphokine interleukin-2 (IL-2) in order to proliferate^{1,2}. Cells that respond to IL-2 possess a surface receptor glycoprotein specific for this lymphokine^{3,4}. We have recently purified and chemically characterized the IL-2 recep-

tor from both phytohaemagglutinin-activated human T cells and the human T-cell lymphoma HUT-102 (ref. 5). From the NH₂-terminal protein sequence obtained in that study, we have now used synthetic oligonucleotides to probe a complementary DNA library, prepared from HUT-102 messenger RNA, for the presence of cDNA clones that might code for the IL-2 receptor. Two cDNA clones were isolated which had closely related DNA sequences. Interestingly, only one coded for an active receptor when transfected into COS-7 cells. This clone contained a 216-base pair (bp) insert that was not present in the other clone. The insert was flanked by an 8-bp direct repeat reminiscent of a transposable element, and appeared to code for a region of marked structural homology to the NH₂-terminal region of the receptor molecule.

Polyadenylated mRNA from HUT-102 cells was purified and used to construct a cDNA library by standard techniques (Figs 1, 2), and oligonucleotide probes corresponding to the amino acid sequence of the IL-2 receptor (Cys-Asp-Asp-Pro-Pro)⁵ were synthesized. As there were 64 possible 17-nucleotide sequences that could encode these amino acids, we carried out primer extension experiments to define a more precise probe (Fig. 1). Of the six oligonucleotide pools tested, only pool N (5'-GGG¹GGGTCGTCGTCACA-3') gave clear primer extension products, of 230 and 280 nucleotides. This pool was therefore used as a probe to screen the HUT-102 cDNA library (Fig. 2).

Two cDNA clones, designated pN1 and pN4, having insert sizes of 1.0 and 2.8 kilobase pairs (kb), respectively, were selected for further analysis. Restriction mapping and cross-hybridization experiments showed that these two clones were closely related, but not identical (Fig. 2a). pN4, in addition to being considerably larger than pN1, contained an insert of about 200 bp not present in pN1. To establish the relationship between these cDNA clones and their possible identity to the IL-2 receptor sequence, we determined their nucleotide sequence (Fig. 2b). Both clones contained the sequence of the oligonucleotide probe N, with a single base pair mismatch, and encoded the amino acid sequence determined for the NH₂-terminus of the IL-2 receptor⁵. They also encoded a stretch of 21 amino acids immediately preceding the NH₂-terminal sequence, starting with methionine and showing many of the characteristics of a hydrophobic signal peptide expected for membrane or secreted proteins.

Both pN1 and pN4 had a single large open reading frame, of 200 and 272 amino acids, respectively, terminating at the same position (nucleotide 861). However, pN4 contained 216 nucleotides not found in pN1 (nucleotides 370-585). This insert was bounded by direct repeats of the 8-bp sequence TTCCAGGT, which was found only once in pN1. Following this insert, the same reading frame was maintained in pN4 and pN1. Only four nucleotide differences were found between the two sequences. These were clustered towards the NH₂-terminal portion of the protein, and were predicted to cause three amino acid replacements, Glu 29 to Lys, Lys 87 to Glu, and Ile 88 to Met. A search of the data bases of the National Biomedical Research Foundation, Genbank and the European Molecular Biology Laboratory detected no significant homologies with other sequences.

To determine whether one or both cDNA clones could encode a functional IL-2 receptor, we attempted their expression in COS-7 monkey kidney cells (Fig. 3). Two plasmids, pN1/N4-S and pN1/N4-X, containing the coding regions of pN4 and pN1, respectively, were transfected into COS-7 cells. The cells were collected 48 h later and assayed for expression of the IL-2 receptor by their ability to bind either ¹²⁵I-labelled IL-2 or 2A3, a monoclonal antibody directed against the IL-2 receptor⁵. We found (Fig. 3b, c) that COS-7 cells transfected with pN1/N4-S could bind specifically both IL-2 and the monoclonal anti-IL-2 receptor antibody, but that neither pN1/N4-X nor mock-transfected COS-7 cells showed any specific binding of either IL-2 or antibody. As pN1/N4-S contains the pN4 coding region, this result indicated that pN4 encodes a functional IL-2 receptor protein, while pN1 does not. The receptor in transfected COS-7 cells migrated on SDS-polyacrylamide gels with a relative

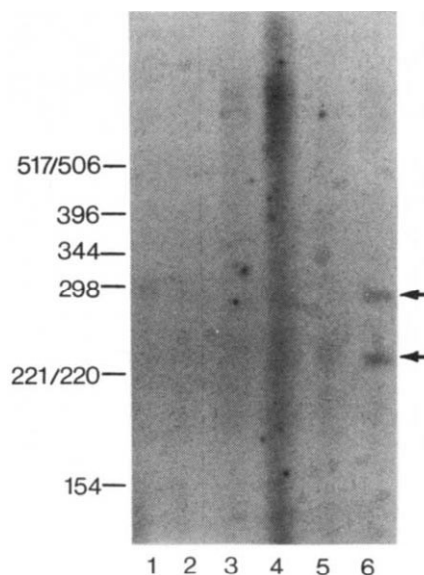


Fig. 1 Primer extension analysis of T-cell RNA. The autoradiogram shows:

lane 1, hybridization with the oligonucleotide pool I, 5'-GGGGG¹TCATCATCACA-3';
lane 2, pool J, GGAGG¹TCATCATCACA;
lane 3, pool K, GGTGG¹TCATCATCACA;
lane 4, pool L, GGCGG¹TCATCATCACA;
lane 5, pool M, GGAGGG¹TCGTCGTCACA;
lane 6, pool N, GG¹GGGTCGTCGTCACA.

M_r markers are ³²P-labelled *Hinf*I fragments of pBR322. In other experiments (data not shown), pool N gave the same primer extension products with RNA from HUT-102 cells, but not with RNA from IL-2 receptor-negative HL60 cells, and was therefore a good candidate for an IL-2 receptor-specific probe.

Methods: Peripheral blood T cells were isolated as described previously¹³ and stimulated for 20 h with the mitogens concanavalin A (20 µg ml⁻¹) and phorbol myristic acetate (10 ng ml⁻¹). Total RNA was isolated by the guanidinium thiocyanate/caesium chloride method¹⁴. Oligonucleotides were ³²P-labelled to a specific activity of 10⁶ c.p.m. per ng with T4 polynucleotide kinase and [γ -³²P]ATP (7,000 Ci mmol⁻¹, NEN). Each probe (5 × 10⁵ c.p.m.) was co-precipitated with 20 µg of RNA, redissolved in 5.5 µl 10 mM Tris pH 8.3, 1 mM EDTA, 4.5 µl 2 M KCl, heated at 65 °C for 5 min, then hybridized at 50 °C for 1 h. The reaction was adjusted to 50 mM Tris pH 8.3, 140 mM KCl, 10 mM MgCl₂, 5 mM dithiothreitol, 100 µg ml⁻¹ actinomycin D, 100 µg ml⁻¹ bovine serum albumin (BSA) and 10 U reverse transcriptase in a final volume of 60 µl. This was incubated at 42 °C for 1 h, followed by the addition of EDTA to 20 mM final concentration. Following phenol:chloroform (1:1) extraction and ethanol precipitation, the nucleic acids were redissolved in 80% formamide, 100 mM Tris-borate pH 8.3, 1 mM EDTA and electrophoresed on a 4% polyacrylamide/8 M urea gel. Following electrophoresis, the gel was dried and exposed to X-ray film.

molecular mass (*M_r*) of 50,000, as detected by Western blot analysis using ¹²⁵I-labelled 2A3 (data not shown).

To study the expression of IL-2 receptor mRNA, we probed Northern blots of RNA from HUT-102 cells and activated peripheral blood T cells (Fig. 4). A probe derived from the *Sst*I-*Bam*HI fragment of pN4 hybridized to two classes of RNA of about 1.4 and 3.4 kb, present in both HUT-102 and T-cell RNA (Fig. 4a). These RNA species were present at less than 1% of these levels in resting T cells (data not shown). Interestingly, a probe specific for the insert in pN4, consisting of the 240-bp *Pst*I-*Bgl*II fragment, hybridized to both RNA classes in HUT-102 and T-cell RNAs (Fig. 4b).

The relationship between pN1 and pN4 remains unknown. pN1 could represent a cloning artefact or an aberrant transcript specific to HUT-102 cells. If pN1 indeed represents a *bona fide* mRNA species, it could be the product of a gene at a locus distinct from, or allelic to, that of pN4. Alternatively, one gene could give rise to both classes of transcripts via alternative RNA

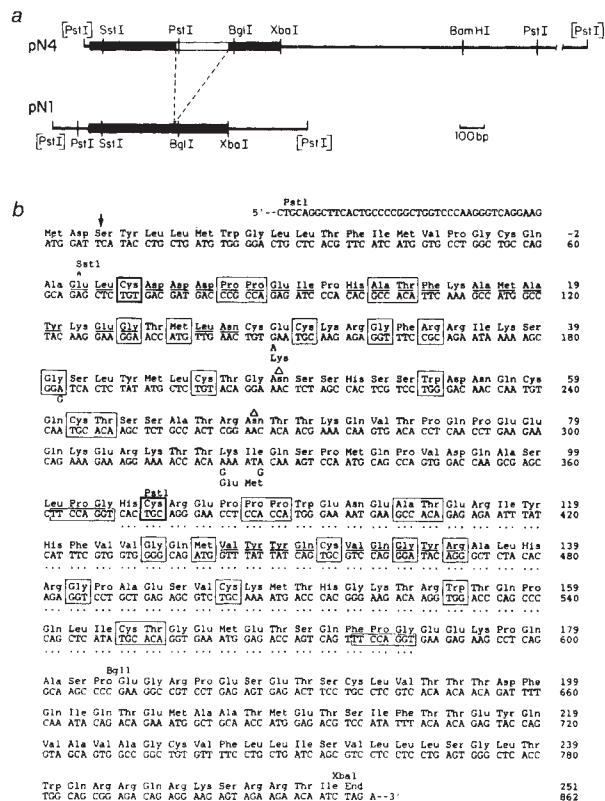


Fig. 2 **a**, Restriction map of pN1 and pN4. *Pst*I sites in brackets are those generated by the cloning procedure. Coding sequences are boxed; the pN4-specific insert is shown as an open box. **b**, The nucleotide sequence and predicted amino acid sequence of the IL-2 receptor. The nucleotide sequence shown is from pN4 except for sequences upstream from the arrow, which are derived from pN1. The arrow marks the 5' end of the insert in pN4. The nucleotides are numbered from the position of the presumed initiator methionine codon, and the amino acids are numbered from the mature NH₂-terminus of the protein, the Glu residue marked with a star. The sequence from pN1 differs from pN4 at nucleotides 148, 183, 322 and 327 (three of which would cause amino acid changes). pN4 also contains a 216-bp insert sequence not present in pN1 (underlined in dots). Underlined amino acids were confirmed by protein sequence analysis and triangles represent possible glycosylation sites. Boxed amino acid and nucleotide sequences indicate homologous residues between the NH₂-terminal region and the insert region when the two heavily boxed residues are aligned, as determined by the computer programs of Needleman and Wunsch²³. The 8-bp repeat flanking the insert is boxed.

Methods: **a**, Double-stranded cDNA was synthesized from HUT-102 polyadenylated RNA by standard procedures¹⁴. The cDNA was size fractionated on a Sephacryl S-400 column and fractions >500 bp in length were pooled and tailed with dCMP residues¹⁵. The tailed cDNA was annealed to dGMP-tailed, *Pst*I-cut pBR322 (NEN) and used to transform *Escherichia coli* strain MM294 according to the procedure of Hanahan¹⁶. Approximately 2×10^6 transformants were obtained from 100 ng of cDNA. Pools of 5,000 transformants were used to make small-scale plasmid DNA preparations¹⁷, digested with *Pvu*II and *Hind*III, electrophoresed on a 0.8% agarose gel, blotted to nitrocellulose filters and hybridized to ³²P-labelled oligonucleotide pool N (10^6 c.p.m. ml⁻¹ in 6×SSC, 0.5% NP40, 0.1% sarcosyl, 5×Denhardt's solution containing 100 μg ml⁻¹ salmon sperm DNA at 50°C for 16 h). Blots were washed extensively in 6×SSC at room temperature and then for 5 min at 55°C before autoradiography. Positive pools were chosen for colony hybridization¹⁸ experiments using the same conditions. Identified transformants which hybridized strongly with the probe were purified. Restriction maps were generated using standard techniques¹⁴. **b**, Plasmids pN1 and pN4 were digested with *Pst*I, *Sst*I, *Xba*I and *Bam*HI in various combinations and cloned with M13mp18 or M13mp19 (ref. 19). DNA sequencing was done by the chain termination method²⁰ as described previously^{21,22}.

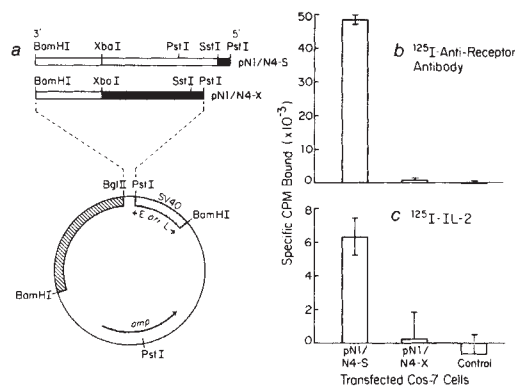


Fig. 3 **a**, Expression of the IL-2 receptor in COS-7 cells. Structure of cDNA expression plasmids. The vector used to express cDNAs (pMLS, shown as a circle) consists of four parts. The stippled box contains the simian virus 40 (SV40) origin of DNA replication, enhancer elements and early and late promoters (SV40 coordinates 5,107–208), originally derived from plasmid pSV2-dhfr (ref. 24) as a *Hind*III–*Pvu*II fragment. Downstream from the early promoter is a synthetic polylinker sequence that includes sites for *Bgl*II and *Pst*I (D.C., unpublished). The hatched box contains the SV40 small-T antigen splice donor and acceptor sites (SV40 coordinates 4,035–4,656) and the SV40 polyadenylation site (SV40 coordinates 2,469–2,706), originally derived from pSV2-dhfr as a *Bgl*II–*Bam*HI fragment²⁴. The thin lines are from the plasmid pML2d, a derivative of pBR322 which lacks sequences inhibitory to DNA replication in mammalian cells^{25,26}. As pN4 lacks the first three amino acids of the presumptive signal peptide, and the presence of dG.cC tails at the 5' end of a cDNA insert can inhibit its expression (ref. 27 and our unpublished observations), we constructed hybrid cDNAs pN1/N4-S and pN1/N4-X, taking advantage of the 'natural' *Pst*I site in the 5' noncoding region of pN1 which lacks tails. Sequences derived from pN1 are shown as solid boxes, and those from pN4 as open boxes. The hybrid cDNA molecules were inserted into the *Pst*I and *Bgl*II sites of the polylinker in pMLS. pN1/N4-S contains the 5' *Pst*I–*Sst*I fragment from pN1 and the *Sst*I–*Bam*HI fragment from pN4, and thus has the coding sequence of pN4. pN1/N4-X contains the 5' *Pst*I–*Xba*I fragment from pN1 and the *Xba*I–*Bam*HI fragment from pN4, and has the coding sequence of pN1 (Fig. 2). **b**, **c**, Transfected COS-7 cells were screened for IL-2 receptor expression by binding of ¹²⁵I-labelled anti-IL-2 receptor antibody 2A3 (**b**) and ¹²⁵I-labelled IL-2 (**c**).

Methods: **a**, Plasmids were transfected into COS-7 cells as described previously^{28,29}. Monolayers of COS-7 cells (10^6 cells per 10-cm plate) were washed twice with Tris-buffered saline (TBS) and exposed to 10 μg of DNA per plate in 1 ml TBS containing 500 μg ml⁻¹ DEAE-dextran (*M*, 5×10^5 , Sigma) for 30 min at room temperature. The cells were washed once more with TBS and fed with growth medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) containing 100 μM chloroquine. After 5 h at 37°C, the medium was replaced by growth medium without chloroquine, and the cells were incubated at 37°C for 48 h, then collected by scraping. **b**, **c**, ¹²⁵I-labelled 2A3 (specific activity 9.8×10^{14} c.p.m. mmol⁻¹) was purified and radiolabelled as described previously⁵. IL-2 was purified by two-step reverse-phase HPLC³⁰ and radiolabelled using the Enzymobead radioiodination reagent (BioRad) essentially following the manufacturer's specifications. Briefly, 50-μl aliquots of IL-2 (5×10^6 U) in acetonitrile and trifluoroacetic acid were combined with 50 μl of 0.2 M sodium phosphate pH 7.2, and the acetonitrile was evaporated under nitrogen. Then 50 μl of Enzymobead reagent, 10 μl of ¹²⁵I (1 mCi) and 10 μg of 2.5% β-D-glucose were added and the mixture was incubated at 25°C for 10 min. Sodium azide (20 μl of 25 mM) and sodium metabisulphite (10 μl of 5 mg ml⁻¹) were then added and after 5 min at 25°C, iodinated IL-2 was separated from unbound ¹²⁵I by chromatography on a 2-ml Sephadex G-25 column equilibrated in 0.05 M sodium phosphate, pH 7.2, containing 0.1% BSA. Based on an initial biological specific activity for IL-2 of 1×10^6 U per μg protein, the radiolabelled preparation had an estimated specific activity of 1×10^{15} c.p.m. mmol⁻¹. Binding assays were performed as described previously³¹. Cells (1.2×10^6) were incubated with either 5×10^{-9} M ¹²⁵I-2A3 or 1.3×10^{-8} M ¹²⁵I-IL-2 in a final volume of 150 μl for 30 min at 37°C. Nonspecific binding was measured in the presence of a 1,000-fold molar excess of unlabelled 2A3 or a 150-fold molar excess of unlabelled IL-2. Replicate 70-μl aliquots of incubation mixtures were centrifuged through phthalate oil, and **b** and **c** show specific c.p.m. bound to separated cell pellets.

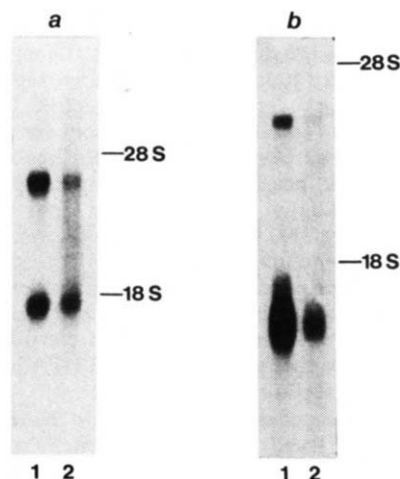


Fig. 4 Northern blot analysis of RNA from HUT-102 and mitogen-stimulated peripheral blood T cells. Poly(A)⁺ mRNA was purified³² from whole-cell RNA, isolated as described in Fig. 1 legend, electrophoresed on 1.5% agarose gels containing formaldehyde and transferred to nitrocellulose filters as described elsewhere¹⁴. Blots were hybridized with ³²P-RNA probes, synthesized by SP6 polymerase as described previously³³. Restriction fragments to be used for probes were subcloned into the pSP64 vector (Promega Biotec). *a*, Probe from the *Sst*I-*Bam*HI fragment of pN4. *b*, Probe from the pN4-specific 240-bp *Pst*I-*Bgl*II fragment of pN4 (Fig. 2). Hybridization was for 16 h at 63 °C in Stark's buffer³⁴. Blots were washed at 68 °C in 0.1 × SSC for 1 h and autoradiographed for 4 h with intensifying screens. Lane 1, 2 µg of poly(A)⁺ whole-cell RNA from HUT-102; lane 2, 2 µg of poly(A)⁺ whole-cell RNA from peripheral blood T cells stimulated as in Fig. 1. The position of large (28S) and small (18S) ribosomal RNAs observed by ultraviolet shadowing of the gel before blotting are indicated.

splicing and/or polyadenylation mechanisms. We are currently attempting to resolve these issues by isolating additional cDNA and genomic clones of the IL-2 receptor gene.

Figure 2*b* shows the sequence coding for a protein of *M_r* 28,428 and defines three structural regions. Region 1 covers residues 1–102, terminating with the first 8-bp repeat. It is potentially rich in carbohydrate, with possible *N*-linked glycosylation sites at Asn residues 49 and 68. *O*-linked glycosylation sites are difficult to predict, but serine- and threonine-rich areas are clustered in the same region around residues 50–54 and 62–66. The *M_r* of the receptor isolated from HUT-102 cells ranges from 47,000 (estimated from the specific activity of the purified receptor of 21,200 fmol of receptor per µg protein⁵) to 55,000 (estimated from the migration of the protein on SDS-polyacrylamide gels⁵). Similar sizes have been reported in studies of radiolabelled receptor^{6–9}. Results of pulse-chase experiments and studies using tunicamycin and glycosidase indicate a protein of *M_r* 33,000–40,000 that is a precursor to the mature protein^{6,9}. Our results suggest that the receptor could easily be composed of 50% carbohydrate.

Region 2 is present only in pN4 and covers residues 103–174, terminating with the second 8-bp repeat. It has a striking homology to region 1 in that if the Cys residue at position 104 is aligned with the Cys at position 3, all of the residues boxed in Fig. 2*b*, including all of the Cys residues, are identical between the two regions. A hydrophilicity plot of the amino acid sequence¹⁰ revealed that this region contains a stretch of 18 largely hydrophobic amino acids (residues 118–135). Because region 2 is absent from pN1 and as pN1/N4-X fails to encode a detectable receptor in transfected COS-7 cells, it is tempting to speculate that this region is responsible for the IL-2 and 2A3 binding properties of the protein encoded by pN4 (pN1/N4-S). It is interesting that the sequence encoding region 2 of pN4 is flanked by 8-bp direct repeats. The generation of small direct repeats enclosing an insertion is characteristic of many types of transposable element¹¹. However, it is unusual for a transposable

element to encode a protein domain that is essential for the function of that protein. We have no evidence that the protein encoded by pN4 is sufficient by itself to deliver a transmembrane signal. It therefore remains possible that it is the IL-2 binding component of a larger IL-2 receptor complex, as previously suggested¹².

The third region consists of the 77 residues extending from the second 8-bp repeat to the C-terminus of the protein and is present in both pN1 and pN4. It contains a presumptive membrane-spanning region of 23 hydrophobic residues between positions 218 and 241, followed by a short, highly basic C-terminal peptide.

The availability of a cDNA encoding the IL-2 receptor should help to elucidate the regulation and function of a protein that is intimately involved in growth control of human T cells.

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1. Morgan, D. A., Ruscetti, F. W. & Gallo, R. C. *Science* **193**, 1007–1008 (1976).
2. Gillis, S. & Smith, K. S. *Nature* **268**, 154–156 (1977).
3. Bonnard, G. D. D., Yosaka, D. & Jacobson, D. J. *Immun.* **123**, 2704–2708 (1979).
4. Robb, R. J., Munck, A. & Smith, K. A., *J. exp. Med.* **154**, 1455–1474 (1981).
5. Urdal, D. L., March, C. J., Gillis, S., Larsen, A. & Dower, S. K. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6481–6485 (1984).
6. Leonard, W. J., Depper, J. M., Robb, R. J., Waldmann, T. A. & Greene, W. C. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6957–6961 (1983).
7. Robb, R. J. & Greene, W. C. *J. exp. Med.* **158**, 1332–1337 (1983).
8. Hemler, M. E. *et al. Hum. Immun.* **8**, 153–165 (1983).
9. Wano, Y. *et al. J. Immun.* **132**, 3005–3010 (1984).
10. Hopp, T. & Woods, K. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3824–3828 (1981).
11. Calos, M. P. & Miller, J. H. *Cell* **20**, 579–595 (1980).
12. Leonard, W. J. *et al. Nature* **300**, 267–269 (1982).
13. Saxon, A., Feldhaus, J. & Robins, R. A. *J. immun. Meth.* **12**, 285–288 (1976).
14. Maniatis, T., Fritsch, E. F. & Sambrook, J. in *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
15. Michaelson, A. M. & Orkin, S. H. *J. biol. Chem.* **257**, 14473–14782 (1982).
16. Hanahan, D. J. *molec. Biol.* **166**, 557–580 (1983).
17. Birnboim, H. C. & Doly, J. *Nucleic Acids Res.* **7**, 1513–1523 (1979).
18. Grunstein, M. & Hogness, D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3961–3965 (1975).
19. Norrander, J., Kempe, T. & Messing, J. *Gene* **26**, 101–106 (1983).
20. Sanger, F., Nicklen, B. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
21. Cerretti, D. P., Dean, D., Davis, G. R., Bedwell, D. & Nomura, M. *Nucleic Acids Res.* **11**, 2599–2616 (1983).
22. Shiba, K., Ito, K., Yura, T. & Cerretti, D. P. *EMBO J.* **3**, 631–635 (1984).
23. Needleman, S. B. & Wunsch, C. D. *J. molec. Biol.* **48**, 443–453 (1970).
24. Subramani, S., Mulligan, R. & Berg, P. *Molec. cell. Biol.* **1**, 854–864 (1981).
25. Lusky, M. & Botchan, M. *Nature* **293**, 79–81 (1981).
26. Sarver, N., Byrne, J. C. & Howley, P. M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7147–7151 (1982).
27. Riedel, H., Kondor-Koch, C. & Garoff, H. *EMBO J.* **3**, 1477–1483 (1984).
28. Luthman, H. & Nagnusson, G. *Nucleic Acids Res.* **11**, 1295–1308 (1983).
29. McCutchan, T. H. & Pagano, J. S. *J. natn. Cancer Inst.* **41**, 351–357 (1968).
30. Urdal, D. L. *et al. J. Chromatogr.* **296**, 171–179 (1984).
31. Dower, S. K., Ozato, K. & Segal, D. M. *J. Immun.* **132**, 751–758 (1984).
32. Aviv, H. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408 (1972).
33. Green, M. R., Maniatis, T. & Melton, D. A. *Cell* **32**, 681–694 (1983).
34. Wahl, G., Stern, M. & Stark, G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683–3687 (1979).

Primary structure of human T-cell receptor α-chain

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The T-cell receptor has been studied intensely over the past 10 years in an effort to understand the molecular basis for major histocompatibility complex (MHC) restricted antigen recognition^{1–9}. The use of anti-receptor monoclonal antibodies to isolate and characterize the receptor from human and murine T-cell clones^{10–15} has shown that the protein consists of two disulphide-linked glycopeptides, α and β, distinct from known

immunoglobulin light and heavy chains. Like immunoglobulin light and heavy chains, however, both the α - and β -chains are composed of variable and constant regions¹⁶⁻¹⁸. Molecular cloning has revealed¹⁹⁻²³ that the β -chain is evolutionarily related to immunoglobulins, and is encoded in separate *V* (variable), *D* (diversity), *J* (joining) and *C* (constant) segments that are rearranged in T cells to produce a functional gene^{19-21,24-27}. We report here cDNA clones encoding the α -chain of the receptor of the human T-cell leukaemia line HPB-MLT. Using these cDNA probes, we find that expression of α -chain mRNA and rearrangement of an α -chain *V*-gene segment occur only in T cells. The protein sequence predicted by these cDNAs is homologous to T-cell receptor β -chains and to immunoglobulin heavy and light chains, particularly in the *V* and *J* segments.

The HPB-MLT α -chain peptides, the corresponding oligonucleotide families and the method used for isolating and subcloning the two α -chain cDNA clones, pGA-1 and pGA-5, are described in detail in Fig. 1 legend. The 570-base pair (bp) *EcoRI*-*PstI* fragment of the pGA-1 insert corresponding to the *V*, *J*, and part of *C* of the α -chain (see Fig. 3) was used to investigate the expression of α -chain mRNA. The fragment was labelled by nick-translation and used to probe mRNA from several T-cell and non-T-cell tumour lines (Fig. 2a). A mRNA species of 1.5 kilobases (kb) was detected in HPB-MLT and in a second T-cell tumour, JURKAT. No mRNA species were detected in the erythroleukaemia line, K562, or in the two B-cell tumour lines U266 and Daudi. Thus the presumptive α -chain mRNA is T-cell-specific and 1.5 kb in length.

The organization of β -chain and immunoglobulin genes coupled with the partial α -chain amino acid sequence²³ predicted that the α -chain should be encoded in gene segments undergoing somatic rearrangements in T cells. Southern blot analysis probing with the 570-bp *V*-*J*-*C* fragment (Fig. 2b) demonstrated the existence of T-cell-specific DNA rearrangements in HPB-MLT and JURKAT but not in two other T-cell lines, CEM and F35.3, or in any of the three non-T-cell lines examined. To determine which portion of the α -chain was encoded on the rearranging fragments in HPB-MLT and JURKAT, we repeated the analysis with separate *V*- and *C*-region probes (Fig. 2b). The *C*-region probe identified a 0.6-kb *PvuII* germ-line fragment and a 5.0-kb *BamHI* germ-line fragment which were not rearranged in any cell line we examined. Similar results were obtained using DNA digested with either *HindIII* or *EcoRI*, indicating that these restriction sites are present in the intron between the *J α* gene segments and the *C α* gene segment. The *V*-region probe detected rearrangements in HPB-MLT and JURKAT, but not in any other cell line, suggesting that HPB-MLT and JURKAT have rearranged the same *V α* gene segment. However, the fact that the rearranged fragments differed in size by ~2 kb may imply a rearrangement to a different *J α* in HPB-MLT and JURKAT. The detection of a single germ-line fragment with this *V α* indicates that it is not a member of a large cross-hybridizing *V α* family such as those often seen among immunoglobulin *V* genes. The failure to detect rearrangement of this *V α* in CEM or F35.3 is not surprising since not all T cells are expected to rearrange and express the same *V* gene. Taken together, these data indicate that, as with other immunoglobulin-like molecules, the α -chain is encoded by separate, non-contiguous segments which somatically rearrange in T cells to produce a functional gene.

The nucleotide sequences of the inserts in pGA-1 and pGA-5 were determined and the portions corresponding to α -chain mRNA deduced (Fig. 3). The two sequences confirmed each other. The α -chain nucleotide sequence and its predicted translated protein (Fig. 3b) shows that of the eight α -chain tryptic peptide sequences which we previously reported²³, seven are present in the predicted protein sequence (A-G), as well as a ninth peptide (I), sequenced but not previously reported. One of the previously-reported peptides (H) is not present. Presumably this peptide was a high yield product of a minor contaminant in our α -chain protein preparation.

As the initiation ATG codon was apparently lost during the

preparation and cloning of these cDNAs, the starting point of the coding sequence is ambiguous. However, the first 20 amino acids shown are very homologous to the hydrophobic leader sequences previously reported for receptor β -chains¹⁹⁻²¹, indicating that only a few bases of coding sequence are missing. Attempts to sequence this α -chain protein have indicated a blocked N-terminus, possibly the result of the presence of a Gln-derived pyrrolidone carboxylic acid (our unpublished observations). Therefore, we suggest that the mature α -chain sequence begins at the first Gln. The rest of the sequence then encodes a 253-amino acid protein of relative molecular mass (M_r) ~ 30,000 (30K) with five possible sites for N-linked glycosylation (Asn-X-Ser or Asn-X-Thr). This is consistent with the known M_r (47 K) of the fully glycosylated HPB-MLT α -chain present on the cell surface^{18,23}.

When the sequence of the mature α -chain protein was compared with that of T-cell receptor β -chains, immunoglobulin light and heavy chains and another T-cell-derived immunoglobulin-like molecule of unknown function (pHDS4)²⁸ areas homologous to *V* region, *J* segment and *C* region could be identified. For the *V* region homologies were most apparent in areas corresponding to the three framework regions of immunoglobulin *V* regions defined by Cys at positions 22 and 91 and the Trp at position 35 (Fig. 4a). The homology in the area just before the second Cys is particularly striking. As we pointed out previously²³, based on a partial α -chain protein sequence, the region corresponding to the *J* segment was strongly homologous to other *J* segments, particularly to two receptor β -chain *J* segments (Fig. 4b). There is no obvious evidence for a *D* segment between the *V* and *J* segments of this α -chain, although this possibility cannot be ruled out without knowledge of the germ-line sequences of the *V* and *J* segments.

The *C*-region of the α -chain contains a domain of 51 amino acids defined by Cys at position 135 and 185. This domain is considerably smaller than the corresponding domain in immunoglobulin chains (61-67 amino acids), T-cell β -chains (62-66 amino acids) or the pHDS4 protein (56 amino acids). The sequence homology to these other proteins in the Cys areas is shown in Fig. 4c. There is some homology to β -chains, but very weak homology to the other proteins. We postulate that the third Cys in the *C*-region is the site of disulphide linkage to the β -chain, as there is a Cys in a corresponding position in β -chain *C* regions. There is no homology to β -chains in this region (Fig. 4c), but some homology to the corresponding area of immunoglobulin heavy-chain hinge regions involved in disulphide linkage to light chains is apparent.

The C-terminal end of the protein contains a hydrophobic stretch of 20 amino acids followed by a final Arg-Trp-Ser-Ser. We postulate that this stretch represents a transmembrane and cytoplasmic segment respectively. The presence of two charged basic amino acids in a transmembrane segment is unusual. A charged basic amino acid is also found in the transmembrane segment of β -chains¹⁹⁻²¹. As the receptor is associated in the membrane with the proteins which make up the T3 complex^{11,29}, we speculate that these basic amino acids may be involved in this interaction.

Our results suggest that the sequence and genetic organization of this T-cell receptor α -chain identify it as a member of the rearranging immunoglobulin-like family of genes. This family now has six identified members: α , β and the pHDS4 protein in T cells and heavy chains; λ and κ in B cells. The dissimilarities among the T-cell- and B-cell-derived sequences indicate their very early evolutionary divergence. This collection of cloned genes may elucidate some fundamental questions on T-cell recognition of antigen, such as the structural basis of MHC restriction of antigen recognition, the selection of the T-cell repertoire in the thymus and the nature of self-tolerance.

Since the submission of this manuscript two additional reports of murine T-cell-derived immunoglobulin-like cDNAs have appeared^{30,31}. The *C*-region of the protein encoded in these cDNAs is very similar to that of the human α -chain reported here. The human and murine sequences are identical at 93 of

Fig. 1 Strategy for cloning the cDNA of the α -chain of the T-cell receptor. Based on a partial amino acid sequence of the T-cell receptor α -chain of HPB-MLT, the three oligonucleotide families shown were synthesized on a Systec DNA synthesizer and purified under the supervision of Dr Bruce Kaplan (Beckman Research Institute, California). A cDNA library was constructed from HPB-MLT in λ gt10³². A total of 5×10^4 recombinant phage were blotted onto nitrocellulose paper (10^4 per 150 mm Petri dish)³³. Duplicate filters were baked at 60 °C for 6 h, washed in $2 \times$ SSC, 10 mM Tris pH 7.5, 0.2% SDS at 65 °C for 4 changes of 1 h each. They were then pre-hybridized in $4 \times$ SSC, $5 \times$ Denhardt's solution, 100 μ g ml⁻¹ sonicated, denatured *E. coli* DNA for 16 h before hybridization. The melting temperatures (T_m) of the members of each family were calculated from the formula $T_m = 2^\circ\text{C}$ (number of dA.dT bp) + 4°C (number of dG.dC.bp)³⁴. For each family the hybridization temperature was the T_m of the member with the lowest T_m . Hybridization was performed for 36 h in the prehybridization solution containing 5 pmol ml⁻¹ of ³²P-5'-end-labelled oligonucleotide mixture at a specific activity of 6×10^6 c.p.m. pmol⁻¹. After hybridization, filters were rinsed three times in $4 \times$ SSC at 4 °C, then washed three times in $4 \times$ SSC at the hybridization temperature for 30 min each. The initial hybridization was performed with oligomer family I. Eight clearly duplicated positive phage were identified. Of these, two were positive with oligomer family II. λ GA-1 tested positive with oligomer family III. Although λ GA-5 failed to hybridize detectably with oligomer family III, it was subsequently shown to contain α -cDNA by hybridization with the insert of λ GA-1 and by its nucleotide sequence. The insert of each phage was subcloned into the *Eco*RI site of pUC13 for subsequent experiments.

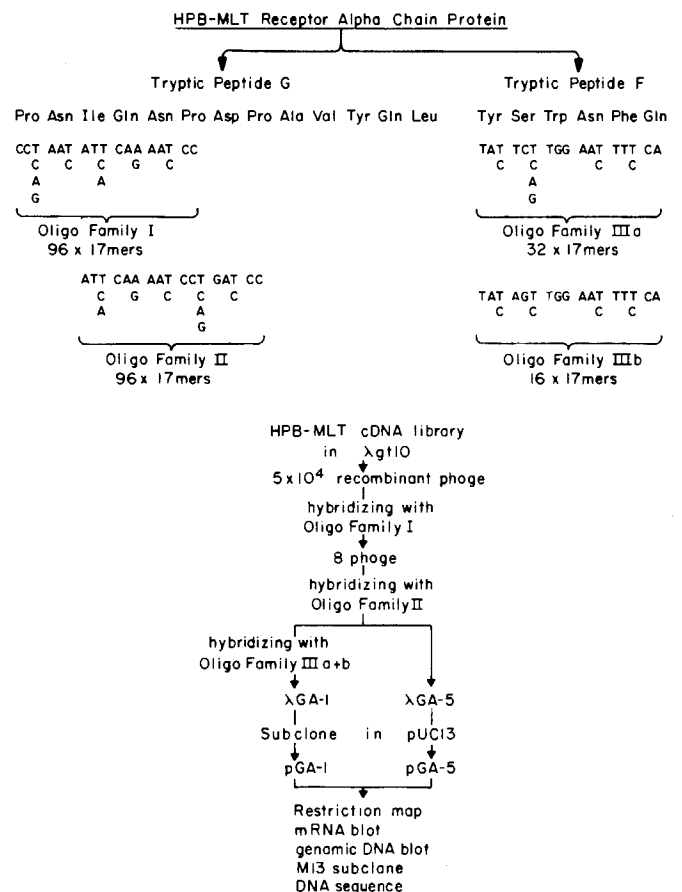
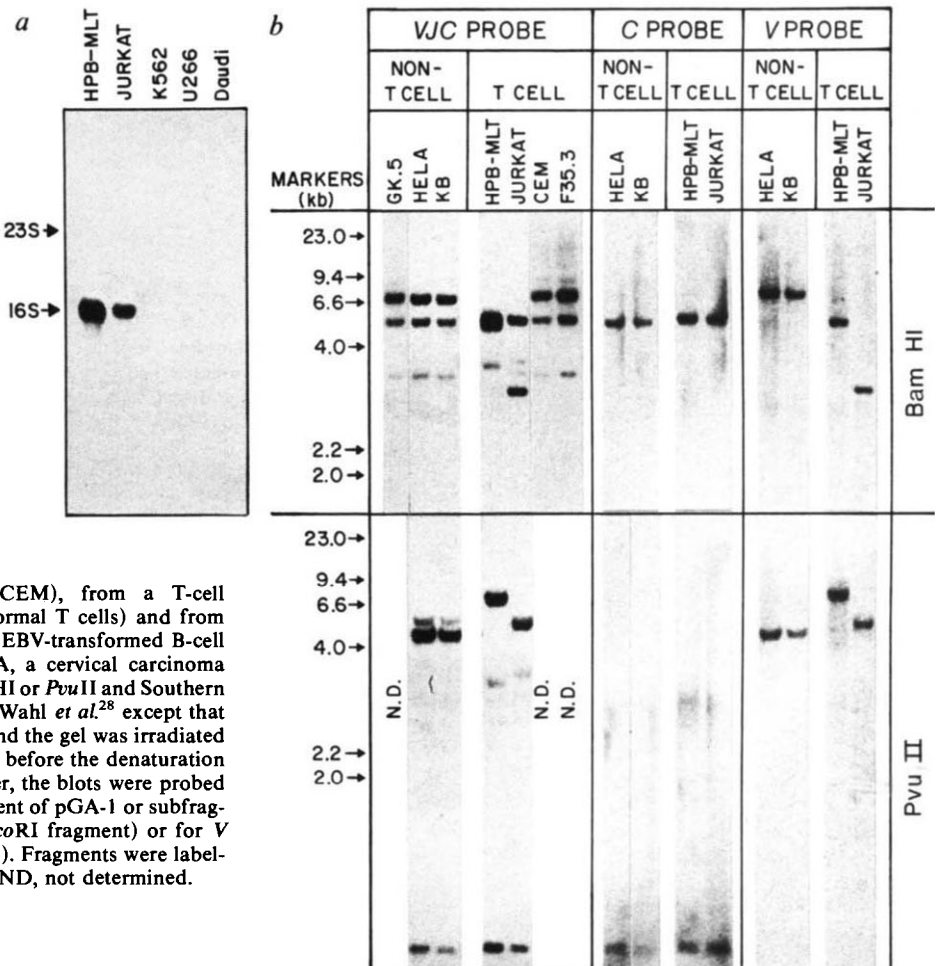


Fig. 2 Expression of α -chain mRNA and rearrangement of α -chain genomic DNA is T-cell-specific. **a**, Poly (A⁺) mRNAs (5 μ g) from two T-cell tumour lines, HPB-MLT and JURKAT, from the erythroleukaemia cell line, K562, the plasmocytoma, U266, and the Burkitt's lymphoma, Daudi, were electrophoresed on a 1% formaldehyde gel and transferred to nitrocellulose paper. The 570-bp *Pst*I-*Eco*RI fragment of the pGA-1 insert (see Fig. 3) was labelled *in vitro* by nick-translation and used as probe. Hybridization was performed in $6 \times$ SSC, 0.2% SDS for 16 h and autoradiography was performed overnight. *Escherichia coli* 16S and 23S rRNA were electrophoresed in parallel as markers. **b**, Genomic DNAs were prepared from three human T-cell tumours (HPB-MLT, JURKAT and CEM), from a T-cell hybridoma (F35.3, fusion of CEM to normal T cells) and from three non-T-cell tumour lines (GK-5, an EBV-transformed B-cell line; KB, a laryngeal carcinoma; HELA, a cervical carcinoma line). The DNAs were digested with *Bam*HI or *Pvu*II and Southern analysis carried out by the procedure of Wahl *et al.*²⁸ except that the acid depurination step was omitted and the gel was irradiated with shortwave ultraviolet light for 8 min before the denaturation step. After transfer to nitrocellulose paper, the blots were probed with either the 570-bp *Pst*I-*Eco*RI fragment of pGA-1 or subfragments specific for C (185-bp *Hae*III-*Eco*RI fragment) or for V (360-bp *Pst*I-*Sau*3a fragment) (see Fig. 3). Fragments were labelled with ³²P *in vitro* by nick-translation. ND, not determined.



142 amino acids in the C-region. This high degree of similarity makes it likely that the murine cDNAs do in fact encode receptor α -chains.

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1. Zinkernagel, R. M. & Doherty, P. C. *Nature* **248**, 701-702 (1974).
2. Bevan, M. *Nature* **269**, 417-419 (1977).
3. Kappler, J. & Marrack, P. *Nature* **262**, 797-798 (1976).
4. Zinkernagel, R. M. *et al. J. exp. Med.* **147**, 882-896 (1978).
5. Kappler, H. W., Skidmore, B., White, J. & Marrack, P. *J. exp. Med.* **153**, 1198-1214 (1981).
6. Hünig, T. & Bevan, B. *J. exp. Med.* **155**, 111-125 (1982).
7. Heber-Katz, E. *et al. J. exp. Med.* **155**, 1086-1099 (1982).
8. Hansburg, D., Heber-Katz, E., Fairwell, T. & Appella, E. *J. exp. Med.* **158**, 25-29 (1983).
9. Marrack, P., Shimonkevitz, R., Hannum, C., Haskins, K. & Kappler, J. *J. exp. Med.* **158**, 1635-1646 (1983).
10. Allison, J., McIntyre, B. & Block, D. *J. Immun.* **129**, 2293-2300 (1982).
11. Meuer, S. *et al. J. exp. Med.* **157**, 705-719 (1983).
12. Haskins, K. *et al. J. exp. Med.* **157**, 1149-1169 (1983).
13. Kaye, J., Porcelli, S., Tite, J., Jones, B. & Janeway, C. A. Jr *J. exp. Med.* **158**, 836-856 (1983).
14. Bigler, R., Fisher, D., Wang, C., Rindoy Kan, E. & Kunkel, H. *J. exp. Med.* **158**, 1000-1005 (1983).
15. Staerz, U., Pasternack, M., Klein, J., Benedetto, J. & Bevan, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1799-1803 (1984).
16. Acuto, O., Meuer, S., Hodgdon, J., Schlossman, S. & Reinherz, E. *J. exp. Med.* **158**, 1368-1373 (1983).
17. McIntyre, B. & Allison, J. *Cell* **34**, 739-746 (1983).
18. Kappler, J. *et al. Cell* **35**, 295-302 (1983).

19. Hedrick, S., Cohen, D., Nielson, E. & Davis, M. *Nature* **308**, 149-153 (1984).
20. Hedrick, S., Nielson, E., Kavaler, J., Cohen, D. & Davis, M. *Nature* **308**, 153-158 (1984).
21. Yanagi, Y. *et al. Nature* **308**, 145-149 (1984).
22. Acuto, O. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 3851-3855 (1984).
23. Hannum, C. J., Kappler, J. W., Trowbridge, I. S., Marrack, P. & Freed, J. *Nature* **312**, 65-67 (1984).
24. Malissen, M. *et al. Cell* **37**, 1101-1110 (1984).
25. Chien, Y., Gascoigne, N., Kavaler, J., Lee, N. & Davis, M. *Nature* **309**, 322-326 (1984).
26. Gascoigne, N., Chien, Y., Becker, D., Kavaler, J. & Davis, M. *Nature* **310**, 387-391 (1984).
27. Siu, G. *et al. Cell* **37**, 393-401 (1984).
28. Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683-3686 (1979).
29. Borst, J., Prendiville, M. A. & Terhorst, C. *J. Immun.* **128**, 1560-1565 (1982).
30. Chien, Y. *et al. Nature* **312**, 31-35 (1984).
31. Saito, H. *et al. Nature* **312**, 36-40 (1984).
32. Huynh, T. V., Young, R. A. & Davis, R. W. in *DNA Cloning Techniques: A Practical Approach* (ed. Glover, D. M.) (IRL Press, Oxford, in the press).
33. Benton, W. O. & Davis, R. W. *Science* **196**, 180-182 (1979).
34. Smith, M. in *Methods of RNA and DNA Sequencing* (ed. Weisman, S. M.) 23-57 (Kraeger, New York, 1983).
35. Messing, J., Crea, R. & Seeburg, P. H. *Nucleic Acids Res.* **9**, 309-317 (1981).
36. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5465 (1977).
37. Saito, H. *et al. Nature* **309**, 757-762 (1984).
38. Kabat, E., Wu, T., Bilofsky, H., Read-Miller, M. & Perry, H. in *Sequences of Proteins of Immunological Interest* (US Department of Health and Human Services, Washington, 1983).

T cell and monocyte-derived burst-promoting activity directly act on erythroid progenitor cells

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While the terminal stages of erythroid differentiation are regulated by the hormone erythropoietin, the early stages of proliferation and differentiation of immature erythroid progenitor cells also depend on cellular factors functionally defined as burst-promoting activity (BPA)¹⁻⁴. Thus, *in vitro* there is suboptimal development of primitive erythroid progenitor cells (burst-forming units—erythroid, BFU-E) into colonies unless a source of BPA is added. It has been demonstrated that T cells⁵⁻⁸ and monocytes⁹⁻¹⁰ produce BPA. Monocytes may represent the main source of BPA and the major role of T cells may be to augment BPA production by monocytes¹⁰. Irradiated bone marrow cells, which contain T cells, monocytes and other BPA-producing cells, also promote BFU-E colony formation¹¹. As these studies used crude BFU-E populations as target cells, it was not possible to define which of the accessory cell products act directly on the progenitor cell. Here we have used a panel of monoclonal antibodies to purify BFU-E from peripheral blood. We demonstrate that BPA produced by both a monocyte and a T-cell line acts directly on the erythroid progenitor cell and can support colony formation by single BFU-E.

Conditioned media containing BPA were obtained from three sources: Mo cells (a T-cell line)¹², giant cell tumour cells (GCTC, a monocytoïd cell line)¹³ and a single batch of human bone marrow conditioned medium. The three conditioned media all have significant levels of BPA, the highest apparent levels being found in the bone marrow conditioned medium. In three experiments the increase in numbers of BFU-E from adherent cell-depleted (Ad⁻), T cell-depleted, peripheral blood mononuclear cells, produced by 10% Mo cell conditioned medium, GCTC and bone marrow conditioned medium was 113, 70 and 133%, respectively. In separate experiments, all three conditioned

media exhibited maximal burst-promoting activity at a concentration of 10% (v/v).

These media were tested against highly purified BFU-E obtained from peripheral blood. Peripheral blood mononuclear cells were cultured from a panel of 15 normal individuals and the three with the highest incidence of BFU-E in the blood were used for later purification procedures. Adherence cell-depleted, T cell-depleted mononuclear cells (Ad⁻E⁻) were prepared from these individuals and cells expressing differentiation antigens characteristic of mature T cells, B cells, monocytes, granulocytes and natural killer (NK) cells were removed by immune-rosetting (Table 1)^{14,15}. The frequency of BFU-E-derived colonies in cultures of the different cell fractions was determined using irradiated bone marrow cells as a source of BPA. In four experi-

Table 1 Monoclonal antibodies used in the purification of BFU-E

Antibody	Specificity
UCHT1*	Pan T
Leu 5†	Pan T
Leu 2a†	'Suppressor' T cells
Leu 3a†	'Helper' T cells
BA1†	B cells and granulocytes
B1‡	B cells
Leu M1†	Monocytes, granulocytes and NK cells
Leu M2†	Some monocytes
Leu M3†	Some monocytes
TG1§	Granulocytes, some monocytes
Leu 7†	NK cells, some T cells

Peripheral blood mononuclear cells from individuals with more than 60 BFU-E per 10⁵ mononuclear cells, were subjected to a rapid (30 min) adherent cell depletion, followed by removal of cells rosetting with 2-aminoethylisothiuronium bromide (AET)-treated sheep red blood cells. The remaining Ad⁻E⁻ cells were incubated with the panel of monoclonal antibodies shown for 30 min at 4 °C. These antibodies react with T cells, B cells, monocytes and NK cells but not with erythroid progenitor cells. The cells were then washed three times and incubated with sheep erythrocytes to which had been coupled affinity-purified rabbit anti-mouse immunoglobulins (Zymed)¹⁴. Cells binding monoclonal antibodies (rosette-forming cells) were then removed on a Ficoll-Hypaque gradient (specific gravity 1.077). In four experiments the mean number of cells forming immune rosettes was 91%. Antibody sources were as follows: * P. C. L. Beverley¹⁷; † Becton Dickinson; ‡ L. Nadler¹⁹ (used only in expts 3 and 4); § P.C.L. Beverley²⁰.

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ments, the incidence of BFU-E-derived colonies in cultures of the Ad⁻E⁻Mab⁻ fraction (Mab⁻, unreactive with monoclonal antibody) was $11.8 \pm 5.1\%$ (Table 2). In experiments 3 and 4 of Table 2, cells expressing HLA-DR antigens were isolated from the Ad⁻E⁻Mab⁻ fraction; the incidence of BFU-E-derived colonies was increased further by more than twofold. In experiments 3 and 4, the DR⁺ progenitor-enriched cell fractions were diluted to 10 cells per ml and plated in 0.1-ml aliquots into microtitre plates; irradiated bone marrow cells were again added. In experiment 3, 16 of 80 wells developed a single BFU-E colony, as did 17 of 96 in experiment 4. The effects of the three conditioned media on these highly purified BFU-E are shown in Fig. 1. There was potent burst-promoting activity in the Mo cell conditioned medium and the GCTC, but only minimal activity in the bone marrow conditioned medium. In the absence of an added source of BPA, the colonies were small (several hundred cells) with poor haemoglobinization. The Mo cell conditioned medium and the GCTC increased both the size of BFU-E (~10,000 cells per BFU-E colony) and their haemoglobin content, as well as the number of colonies formed. In concurrent experiments using crude Ad⁻E⁻ peripheral blood leukocytes as targets, the bone marrow conditioned medium had BPA activity comparable with that seen in the Mo cell conditioned medium and the GCTC.

To confirm that the Mo cell conditioned medium and GCTC acted directly on the progenitor cells, purified progenitor cells from experiment 4 were also cultured in microwells (1 cell per well) by the method of limiting dilution. Table 3 shows that Mo cell conditioned medium and GCTC were indeed able to support the development into colonies of BFU-E grown at these concentrations, approaching one nucleated cell per well.

These results clearly show that the products of human T-cell and monocyte cell lines exert their burst-promoting activities directly on the erythroid progenitor cells, but do not indicate whether these activities are derived from the same molecular species. It is a reasonable assumption that fresh T cells and monocytes also produce directly acting BPA. Established cell lines were used in this study to overcome the potential difficulties with minor cell contaminants in freshly prepared cell preparations.

It is of considerable interest that the batch of bone marrow conditioned medium used in this study, which had high levels of apparent burst-promoting activity, failed to stimulate colony growth by purified BFU-E and therefore must act indirectly to enhance progenitor cell growth in the impure BFU-E populations. Clearly, other batches of bone marrow conditioned

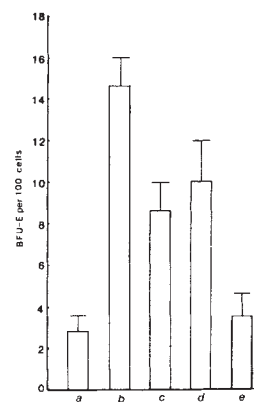


Fig. 1 Effect of irradiated bone marrow cells and conditioned media on the growth of BFU-E from highly purified cell fractions. *a*, No addition; *b*, irradiated bone marrow cells; *c*, Mo cell conditioned medium; *d*, GCTC; *e*, bone marrow conditioned medium. Purified progenitors were cultured in methylcellulose in the presence of 2 units of erythropoietin ml^{-1} as described in Table 2. Irradiated bone marrow cells were added at $2.5 \times 10^4 \text{ ml}^{-1}$. Mo cell conditioned medium was given by Dr D. Golde; GCTC was obtained from Gibco; the bone marrow conditioned medium was made by incubating normal low-density bone marrow cells at $2 \times 10^6 \text{ cells ml}^{-1}$ in IMDM plus 10% FCS for 6 days. Conditioned media were all added to a final concentration of 10% (v/v). Conditioned medium from another T-cell line (Jurkatt) was also tested in two experiments and showed no burst-promoting activity against these purified progenitor cells.

medium may have predominantly directly acting BPA, but great caution must be exercised when preparing burst-enhancing factors from such a heterogeneous cell source. The Mo cell conditioned medium and GCTC may also contain indirectly acting factors but this was not determined in our study. It is difficult to ascertain the significance of indirectly acting factors which enhance BPA production, but it has been shown that the serum from aplastic anaemia patients increases leukocyte BPA production compared with normal serum¹⁶. The cellular origin of indirectly acting burst-enhancing factors such as this is unknown. T cells are certainly one possible source¹⁰. We have shown previously that bone marrow contains three populations of cells which enhance BFU-E growth: T cells, monocytes and a third population of lymphoid cells which express Fc receptors for IgG¹¹. It is unknown whether this third accessory cell popula-

Table 2 Purification of BFU-E from peripheral blood

Cell fraction	No. of BFU-E per 10^5 cells plated			
	Expt 1	Expt 2	Expt 3	Expt 4
F/H	133.5	75	77	76
Ad ⁻ E ⁻	777	322	282	180
Ad ⁻ E ⁻ Mab ⁻	17,228	14,200	10,500	5,286
Ad ⁻ E ⁻ Mab ⁻ DR ⁺	—	—	23,200	16,600

The number of BFU-E per 10^5 cells is shown for each cell fraction. F/H, Mononuclear cells prepared by Ficoll-Hypaque density separation from whole blood. BFU-E colonies were grown in 0.9% methylcellulose in Iscove's modified Dulbecco's medium (IMDM; Gibco) with 30% fetal calf serum (FCS), 0.9% deionized bovine serum albumin (BSA), 10^{-4} M β -mercaptoethanol and 2 U ml^{-1} crude erythropoietin (Connaught Step III)²¹. Irradiated bone marrow cells (3×10^5) served as a potent source of BPA. Irradiated bone marrow cells cultured alone gave rise to no BFU-E colonies. In experiments 3 and 4, the antibody-negative cells (Mab⁻) (<3% positive) were subsequently reacted with a monoclonal antibody^{1,2} to HLA-DR²² and immune rosettes formed. The cells were then fractionated on isotonic Percol (pH 7.4, specific gravity 1.085), and the DR-positive (DR⁺) cells in the pellet were freed of red cells by ammonium chloride lysis. Cells from the final two fractions were plated at $5 \times 10^2 \text{ ml}^{-1}$. The yield of BFU-E in these experiments varied between 21 and 45%. Some of these losses (mean 18%) could be readily accounted for by cells removed for cell counting.

Table 3 BFU-E growth from progenitor-enriched fraction

Additive	No. of wells plated	No. of BFU-E
None	94	0
SRBC	95	0
SRBC+Mo cell conditioned medium	95	5
SRBC+GCTC	95	7
SRBC+irradiated bone marrow	92	17

The growth of BFU-E colonies from highly purified progenitor cell populations, plated at a concentration approaching 1 cell per well, depends on the addition of Mo cell conditioned medium, GCTC or irradiated bone marrow cells. Note that the BFU-E growth in microwells was slower than that in Petri dishes and the BFU-E attained a smaller final size. The culture medium was identical to that described in Table 2 except that 2×10^5 washed sheep red blood cells (SRBC) were added to each well as previous experiments had shown that, at very low cell numbers, the growth of BFU-E in microwells was very poor without some other source of 'filler' cells. Red cells were chosen as these are inert and contain no BPA activity: for example, in experiment 4, the number of BFU-E growing from the progenitor-enriched fraction plated at $5 \times 10^2 \text{ ml}^{-1}$ in 1-ml Petri dishes was 1,200 per 10^5 without any added cells or medium, 1,300 per 10^5 with sheep red blood cells added and 16,600 per 10^5 with irradiated bone marrow cells.

tion produces directly or indirectly acting factors—further studies are required to address this issue.

The study reported here shows that BPA produced by a T-cell and a monocyte cell line acts directly on committed erythroid progenitors whereas other bone marrow-derived factors may act indirectly; it illustrates the complexity of cell interactions involved in the regulation of erythropoiesis, and underlines the importance of using pure populations of progenitor

cells to elucidate these events. The purity of BFU-E obtained in this study is considerably greater than that described previously^{17,18} and this should facilitate further understanding of the regulation and differentiation of erythroid progenitor cells.

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1. Iscove, N. N. *Cell Tissue Kinetics* **10**, 323–334 (1977).
2. Mara, H. & Ogawa, M. *Expl Haemat.* **5**, 141–148 (1977).
3. Iscove, N. N. *ICN-UCLA Symp. molec. cell. Biol.* Vol. **7**, 37–82 (1978).
4. Tsang, R. W. & Aye, M. T. *Expl Haemat.* **7**, 383–388 (1979).
5. Nathan, D. G. *et al. J. exp. Med.* **147**, 324–329 (1978).
6. Mangan, K. F. & Desforges, J. F. *Expl Haemat.* **8**, 717–727 (1980).
7. Torok-Storb, B. J., Martin, P. J. & Hansen, J. A. *Blood* **58**, 171–174 (1981).
8. Reid, C. D. L., Baptista, L. C. & Chanarin, I. *Br. J. Haemat.* **58**, 171–174 (1981).
9. Gordon, L. I., Miller, W. J., Branda, R. F., Janjani, E. D. & Jacob, H. S. *Blood* **55**, 1047–1050 (1980).
10. Zuckerman, K. S. *J. clin. Invest.* **67**, 702–709 (1981).
11. Linch, D. C., Lipton, J. M. & Nathan, D. G. *J. clin. Invest.* (submitted).

12. Golde, D. W., Bersch, N., Quan, S. G. & Lusis, A. J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 593–596 (1980).
13. Abboud, C. N., Brennan, J. K., Barlow, G. H. & Lichtman, M. A. *Blood* **58**, 1148–1154 (1981).
14. Indivieri, F., Wilson, B. S., Pellegrino, M. A. & Ferrone, S. *J. immun. Meth.* **29**, 101–104 (1979).
15. Griffin, J. D., Beveridge, P. & Schlossman, S. F. *Blood* **60**, 30–35 (1982).
16. Nissen, C., Moser, Y. & Speck, B. *Br. J. Haemat.* **51**, 385–390 (1982).
17. Beverley, P. C. L. & Callard, R. F. *Eur. J. Immun.* **11**, 329–334 (1981).
18. Wisniewski, D., Platsoucas, C., Strife, A., Lambek, C. & Clarkson, B. *Expl Haemat.* **10**, 817–829 (1982).
19. Nadler, L. M. *et al. J. Immun.* **126**, 1941–1947 (1981).
20. Beverley, P. C. L., Linch, D. C. & Delia, D. *Nature* **287**, 332 (1980).
21. Iscove, N. N., Sieber, F. & Winterhalter, K. H. *J. cell. Physiol.* **83**, 309–320 (1974).
22. Nadler, L. M. *et al. Hum. Immun.* **1**, 77–90 (1980).

Variant (6;15) translocation in a murine plasmacytoma occurs near an immunoglobulin κ gene but far from the *myc* oncogene

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Chromosome translocations in B-lymphoid tumours are providing intriguing insights and puzzles regarding the role of immunoglobulin genes in the activation of the *myc* oncogene (reviewed in refs 1, 2). The 15;12 translocations found in most murine plasmacytomas and the analogous 8;14 translocation in human Burkitt's lymphomas involve scissions of murine chromosome 15 (human chromosome 8) near the 5' end of the *c-myc* gene and subsequent fusion near an immunoglobulin heavy-chain gene. The less well characterized 'variant' translocations found in about 15% of such tumours also involve the *myc*-bearing chromosome band, but exchange occurs with a chromosome bearing an immunoglobulin light-chain locus—in mice, the κ -chain locus bearing chromosome 6 (refs 3–5) and, in man, chromosome 2 (or 22), at the same band at which the κ (or λ) locus lies (reviewed in ref. 1). The Burkitt variant translocations involve scissions 3' of *c-myc*^{6–10}; one 8;22 translocation placed the C_{λ} locus just 3' of *c-myc*¹⁰, but usually the chromosome 8 breakpoint is a greater, but unknown, distance away from *c-myc*, more than 20 kilobases (kb) in one 8;2 translocation involving the C_{κ} gene⁹. Little is known about the murine 6;15 translocations, although a C_{κ} gene cloned from one plasmacytoma (PC7183) is linked, via chromosome 12 sequences, to an unidentified region of chromosome 15 (ref. 11). We describe here the chromosome fusion region from plasmacytoma ABPC4, which displays the typical reciprocal 6;15 translocations⁵. We find that the chromosome 6 breakpoint is near C_{κ} but, unlike those in the heavy-chain locus, not at a position where immunoglobulin genes normally recombine. Moreover, the chromosome 15 sequences involved in the ABPC4 translocation are not derived from the vicinity of *c-myc*.

Because the chromosome 15 breakpoint in a 15;6 translocation is cytogenetically indistinguishable from that in a 15;12 translocation^{3–5}, we initially expected to find a rearranged *c-myc* gene in ABPC4. However, Southern blot hybridization revealed no alteration within the 20.5-kilobase (kb) *EcoRI* fragment bearing *c-myc* in ABPC4, nor in several other lines with a 15;6 translocation (refs 5, 12 and our unpublished results). Pursuing the hypothesis that the 15;6 translocation generates breaks near C_{κ} , we cloned both rearranged C_{κ} alleles from this κ -expressing tumour. The rearrangement on one allele proved to be within

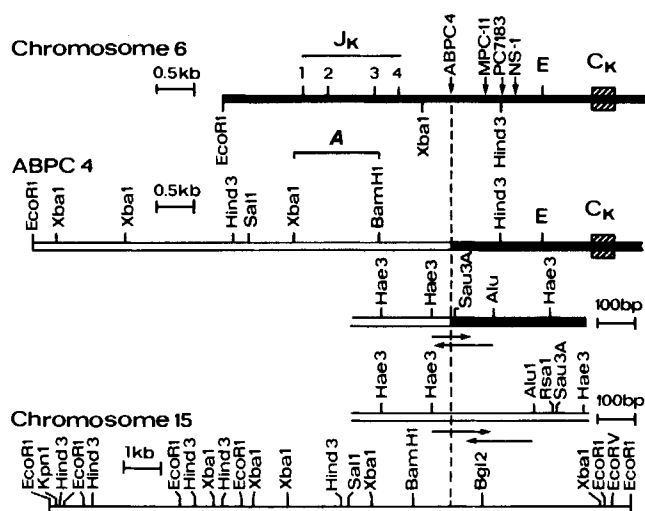


Fig. 1 Restriction endonuclease maps of the germ-line J_{κ} - C_{κ} locus, the ABPC4 C_{κ} gene linked to a chromosome 15 region and the corresponding unrearranged chromosome 15 region. The C_{κ} locus is depicted by a filled bar and chromosome 15 sequences by an open bar. Vertical arrows in the J_{κ} - C_{κ} intron mark points of recombination in ABPC4 and in three other plasmacytomas (see text). E denotes the κ enhancer and A marks the region used as a probe. On the expanded maps, arrows denote the direction and extent of sequencing (see Fig. 3). The hatched box indicates the C_{κ} exon.

Methods: ABPC4 was induced in a BALB/c mouse by pristane and infection with Abelson murine leukaemia virus, which accelerates plasmacytoma formation⁵. Southern blots of ABPC4 DNA with a C_{κ} probe revealed two rearranged *EcoRI* fragments (14.5 and 16.9 kb). Fragments of those sizes, enriched on glycerol gradients, were cloned in phage vector Charon 4A. The 14.5-kb fragment (not shown) presumably bears the active $V_{\kappa}J_{\kappa}C_{\kappa}$ gene in this κ -expressing tumour, as its rearrangement proved to be within the J_{κ} region, in contrast to the intron rearrangement in the 16.9-kb fragment, the 5' portion of which is shown above. When the DNA rearranged to the J_{κ} - C_{κ} intron proved to originate from chromosome 15 (see text), probe A from the chromosome 15 portion was used to isolate the corresponding region of unrearranged chromosome 15 DNA from a library of BALB/c embryonic DNA fragments made by partial *EcoRI* digestion.

the κ -chain joining (J_{κ}) locus, presumably reflecting conventional V_{κ} - J_{κ} recombination. On the other allele, however, rearrangement occurred within the J_{κ} - C_{κ} intron (Fig. 1). The incoming DNA was shown to be derived from chromosome 15 by hybridizing probe A in Fig. 1 to DNA from a panel of mouse-hamster hybrid lines^{13–15} used previously, in collaboration with Dr Uta Francke, to assign *c-myc* to chromosome 15 (ref. 15). The probe hybridized to DNA of the same lines as a

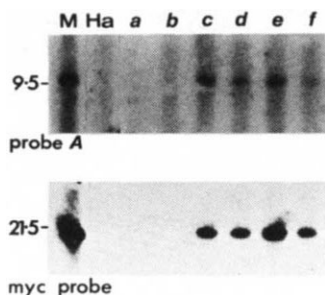


Fig. 2 Blot hybridization data from mouse-hamster cell lines showing that DNA fused to the C_{κ} locus in ABPC4 is derived from chromosome 15. The Southern blots show hybridization with a probe derived from the incoming DNA (A in Fig. 1) and a *c-myc* probe to DNA from BALB/c mouse embryos (M), hamster liver (Ha) and six mouse-hamster cell lines selected to be informative for chromosome 15¹³⁻¹⁵. The sizes of the hybridizing mouse fragments are in kilobases. The cell lines, the full karyotype of which was determined by Francke and colleagues^{13,14} and provided in Table 1 of ref. 15, were as follows: a, I-18A HAT; b, I-18A-2a-8AG; c, EBS 58; d, EBS 11; e, EBS 4; and f, I-13A-Ia-8AG. Mouse chromosome 6 is present in good yield in the DNA in lanes a and f, low in b and c and absent in lanes d and e (ref. 15); hence, the results in lanes a, d and e and probably c are inconsistent with derivation of probe A sequences from chromosome 6.

c-myc probe (Fig. 2). The complement of mouse chromosomes in these lines, specified in Table 1 of ref. 15, eliminates derivation of probe A sequences from any mouse chromosome except 15. In particular, results with at least three lines are inconsistent with its being chromosome 6 (see legend to Fig. 2). We conclude that the ABPC4 clone in Fig. 1 bears the 15; 6 fusion region.

The κ locus has previously been assigned to chromosome 6 (ref. 16), but has been localized only by genetic linkage data¹⁷. As cytogenetic studies³⁻⁵ place the chromosome 6 breakpoint at band C2, our evidence that C_{κ} lies at the breakpoint maps the κ locus to that band, placing κ about 0.4 of the distance from the centromeric end to the distal end of the chromosome, in good agreement with the linkage map¹⁷.

To analyse the nature of the recombining sequences, we determined sequences across the 15; 6 fusion point in ABPC4 and across a corresponding region of unrearranged chromosome 15 DNA cloned from embryos (Fig. 1, bottom). Figure 3 compares these sequences with that in the J_{κ} - C_{κ} intron¹⁸. Recombination occurred 1.85 kb 5' to C_{κ} , that is, 665 base pairs (bp) 3' to the nearest J_{κ} gene. Like the 15; 12 translocation¹⁹⁻²¹, this exchange clearly represents nonhomologous recombination, as the chromosome 15 sequence is not related to the J_{κ} - C_{κ} sequence, except for an AT at the recombination point in both. No repeated sequence elements seem to be involved, as the region around the chromosome 15 (and 6) breakpoints behave as unique sequences in hybridization experiments (Fig. 2 and other blots not shown). Significantly, the heptamer and nanomer 'signal sequences' required for V_{κ} - J_{κ} fusion (reviewed in refs 22, 23) do not occur in the chromosome 15 region sequenced, nor in this portion of the J_{κ} - C_{κ} intron—the heptamer at which a V_{κ} gene aberrantly recombined in MPC-11 (ref. 24) lies 440 bp 3' to the ABPC4 breakpoint (Fig. 1, top). Because V_{κ} - J_{κ} recombination always seems to occur within a few base pairs of the heptamer signal, it seems unlikely that V - J joining enzymes catalysed this 6; 15 translocation or the two best-characterized

examples of Burkitt variant translocations, which involve break-points 5' to the J_{κ} (ref. 10) and J_{κ} (ref. 9) regions. Thus, whereas the immunoglobulin switch recombination machinery may well participate in the 15; 12 translocation, particularly in the chromosome 12 scissions²¹, there is no indication that immunoglobulin rearrangement enzymes are involved in the variant translocations.

The ABPC4 translocation differs in several respects from the bizarre product involving sequences from chromosomes 15, 12 and 6 found in plasmacytoma PC7183 (ref. 11). While both involve a chromosome 6 breakpoint within the J_{κ} - C_{κ} intron, that in PC7183 is several hundred base pairs closer to C_{κ} (Fig. 1, top). More importantly, the cloned chromosome 15 region involved in the PC7183 translocation¹¹ (kindly provided by Drs M. Weigert and M. Shapiro) is not related to the 17-kb segment of chromosome 15 described here (Fig. 1, bottom), as their restriction maps differ and the PC7183 probe did not hybridize to the clones in Fig. 1. Thus, variant translocations can involve different chromosome 15 breakpoints. The most dramatic difference between the ABPC4 and PC7183 translocations, however, is that whereas sequences from chromosomes 15 and 6 directly abut in ABPC4 (Fig. 3), in PC7183 they are separated by 1 kb of the immunoglobulin heavy-chain locus (S_{μ} region)¹¹ from chromosome 12. Thus, PC7183 must have undergone multiple recombination events, whereas ABPC4 represents a simple exchange. From the cytogenetics of tumours like ABPC4³⁻⁵, one would expect a reciprocal translocation product in ABPC4, and indeed we have detected such a product, which will be described elsewhere.

The region 5' to the C_{κ} gene seems to be prone to aberrant recombination events. An atypical translocation linking C_{κ} to chromosome 10 has been described in plasmacytoma NS-1 (ref. 25) and its breakpoint lies only 180 bp from that in PC7183 (Fig. 1, top). In another plasmacytoma, an interstitial A-particle element has become inserted ~0.1 kb 5' to the ABPC4 breakpoint²⁶, while two others have deletions across C_{κ} which end within the J_{κ} - C_{κ} intron²⁷. These findings suggest that the J_{κ} - C_{κ} region is a recombinational 'hot spot' in B lymphocytes. This propensity might be related to its involvement in V_{κ} - J_{κ} assembly, to the constitutive C_{κ} expression in B cells²⁸ and/or to the nuclease hypersensitivity²⁹⁻³² associated with the C_{κ} enhancer region (see below).

The *c-myc* gene is transcribed in ABPC4 at levels comparable to that in other plasmacytomas (refs 5, 12 and our unpublished results). As only the *c-myc* allele involved in a translocation is active in most lymphoid tumours^{6,7,20,34,35}, it seems very likely that the 15; 6 translocation has induced *myc* expression in ABPC4. Hence, it is intriguing that the ABPC4 breakpoint is not near the *myc* gene. Comparison of the 17-kb segment of chromosome 15 defined in Fig. 1 (bottom) with clones spanning 20.5 kb of the *c-myc* locus¹⁹ places the ABPC4 breakpoint more than 20 kb from the *c-myc* promoters, and other clones (our unpublished results) place it more than 50 kb away, depending on whether it lies 5' or 3' of *c-myc*. In striking contrast, the vast majority of 15; 12 translocations break the *c-myc* transcriptional unit¹⁹. This marked difference may mean that the 15; 12 and 15; 6 translocations activate *myc* by different mechanisms.

Translocation to C_{κ} would be favoured in a B lymphocyte if the C_{κ} region induces expression of an incoming gene, as it does for the V_{κ} gene³⁶. Induction of V_{κ} transcription requires proximity to the enhancer (E in Fig. 1) located 0.6 kb 5' to C_{κ}

Ch15 AAAGCAACCTAGGCTCTGGTCTGGTTATGGAGACACTCTGTTTGGCCCTCCGCTCATTGCAATGACAAATTATTATCCTTGGCTCAGGATAAAATTTCTCAGAGTACGGATACGAGAAGTTCAAGGACAAAGATTAAACAGT
A4 AAAGCAACCTAGGCTCTGGTCTGGTTATGGAGACACTCTGTTTGGCCCTCCGCTCATTGCAATGACAAATTATTATCCTTGGCTCAGGATAAAATTTCTCAGAGTACGGATACGAGAAGTTCTATTCTAAATTTGTC
C_κ TGTCCTCTAAGTTTATGACTACAAAAATCAGTAGTAGTCTGAAATATCAATTAAGCTGTTTGAAGTATGACTGCTTGCATGATAGATACCATGGCTGTCTGAATAATCAGAAGAGGTGTGACTCTTATTCTAAATTTGTC

Fig. 3 Sequences involved in the 6; 15 translocation in ABPC4. Chromosome 6 sequences are given in italics and chromosome 15 in standard typeface. Due to the AT on both chromosomes at the recombination point, cross-over could have occurred between any of three residues, as indicated. The chromosome 15 G residue marked with an asterisk has been converted to an A residue in ABPC4, presumably as a consequence of the translocation, but no other alterations were found from the germ-line chromosome 15 or chromosome 6 sequence.

Methods: Detailed restriction maps near the junction (Fig. 1) localized the recombination points, and appropriate fragments were inserted into the single-stranded phage vector M13mp10 or M13mp11 (ref. 39). Sequences were determined by the dideoxynucleotide method⁴⁰.

(refs 37, 38). A direct effect of the C_{κ} enhancer on *c-myc* transcription in ABPC4 appears unlikely, however, because of the large distance involved. Nevertheless, the C_{κ} locus might generate long-range effects along the translocated chromosome (in *cis*) by perturbation of the chromatin suprastructure. Alternatively, the variant translocation might induce *myc* expression in *trans* through an effect on a gene regulating *myc* expression.

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1. Klein, G. *Cell* **32**, 311-315 (1983).
2. Perry, R. *Cell* **33**, 647-649 (1983).
3. Ohno, S. *et al. Cell* **18**, 1001-1007 (1979).
4. Wiener, F. *et al. J. exp. Med.* **159**, 276-291 (1984).
5. Ohno, S. *et al. J. exp. Med.* **159**, 1762-1777 (1984).
6. Erikson, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 7581-7585 (1983).
7. Croce, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 6922-6926 (1983).
8. Davis, M., Malcolm, S. & Rabbitts, T. *Nature* **308**, 286-288 (1984).
9. Taub, R. *et al. Cell* **37**, 511-520 (1984).
10. Hollis, G. *et al. Nature* **307**, 752-755 (1984).

11. Van Ness, B. *et al. Nature* **301**, 425-427 (1983).
12. Adams, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 6966-6970 (1982).
13. Taggart, R., Tetri, P. & Francke, U. *Genet. Cell Genet.* **6**, 769-776 (1980).
14. Francke, U., Tetri, P., Taggart, R. & Oliver, N. *Cytogenet. Cell Genet.* **31**, 58-69 (1981).
15. Cory, S. *et al. EMBO J.* **2**, 213-216 (1983).
16. Hengartner, H., Meo, T. & Müller, E. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4494-4498 (1978).
17. Roderick, T. & Davidson, M. *Mouse News Lett.* **69**, 3-10 (1983).
18. Max, E., Maizel, J. & Leder, P. *J. biol. Chem.* **256**, 5116-5120 (1981).
19. Cory, S., Gerondakis, S. & Adams, J. *EMBO J.* **2**, 697-703 (1983).
20. Bernard, O., Cory, S., Gerondakis, S., Webb, E. & Adams, J. *EMBO J.* **2**, 2375-2383 (1983).
21. Gerondakis, S., Cory, S. & Adams, J. *M. Cell* **36**, 973-982 (1984).
22. Adams, J. & Cory, S. in *Eukaryotic Genes* (eds Maclean, N., Gregory, S. & Flavell, R.) 343-364 (Butterworths, London, 1983).
23. Tonegawa, S. *Nature* **302**, 575-581 (1983).
24. Seidman, J. G. & Leder, P. *Nature* **286**, 779-783 (1980).
25. Perlmutter, R., Klotz, J., Pravtcheva, D., Ruddie, F. & Hood, L. *Nature* **307**, 473-476 (1984).
26. Hawley, R., Shulman, M., Murialdo, H., Gibson, D. & Hozumi, N. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7425-7429 (1982).
27. Durdik, J., Moore, M. & Selsing, E. *Nature* **307**, 749-752 (1984).
28. Van Ness, B. *et al. Cell* **27**, 593-602 (1981).
29. Weischet, W., Glotov, B., Schnell, H. & Zachau, H. *Nucleic Acids Res.* **10**, 3627-3645 (1982).
30. Parslow, T. & Granner, D. *Nature* **299**, 449-451 (1982).
31. Chung, S.-Y., Folsom, V. & Wooley, J. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2427-2431 (1983).
32. Parslow, T. & Granner, D. *Nucleic Acids Res.* **11**, 4775-4792 (1983).
33. Mushinski, J., Potter, M., Bauer, S. & Reddy, E. *Science* **220**, 795-798 (1983).
34. Adams, J., Gerondakis, S., Webb, E., Corcoran, L. & Cory, S. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1982-1986 (1983).
35. Leder, P. *et al. Science* **222**, 765-771 (1983).
36. Mather, E. & Perry, P. *Nucleic Acids Res.* **9**, 6855-6867 (1981).
37. Queen, C. & Stafford, J. *Molec. Cell Biol.* **4**, 1042-1049 (1984).
38. Picard, D. & Schaffner, W. *Nature* **307**, 80-82 (1984).
39. Messing, J., Crea, R. & Seeburg, P. *Nucleic Acids Res.* **9**, 309-322 (1980).
40. Sanger, F., Coulson, A., Barrell, B., Smith, A. & Roe, B. *J. molec. Biol.* **143**, 161-178 (1980).

Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells

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The effects of steroid hormones are mediated by intracellular hormone-specific receptor proteins; the interaction between the hormone and its receptor increases the affinity of the receptor for nuclear binding sites, thereby modulating the expression of specific genes¹. The glucocorticoid receptor is a soluble protein of relative molecular mass (M_r) 94,000 (94K), present at a low relative abundance ($\leq 0.01\%$); it has been purified to near-homogeneity^{2,3}, and specific antisera and monoclonal antibodies have been produced⁴⁻⁶. Purified glucocorticoid receptor binds *in vitro* with high affinity to defined regions of DNA near regulated promoters⁷⁻¹⁰, and sequences essential for these interactions are functional *in vivo* as hormone-dependent transcriptional enhancer elements^{11,12}. We have now cloned complementary DNA (cDNA) for the rat liver glucocorticoid receptor and we describe here a 2.6-kilobase (kb) receptor cDNA isolated following polysome immuno-enrichment of receptor messenger RNA with glucocorticoid receptor-specific antibodies. The receptor appears to be encoded by a single-copy gene which specifies a ~6-kb transcript in rat and mouse cells; this mRNA is altered quantitatively and qualitatively in several mutant cell lines with specific defects in receptor function.

Polysomes and RNA were isolated from J2.17 cells, a mammary tumour virus (MTV) infected derivative of the rat hepatoma HTC line¹³. Figure 1 compares *in vitro* translation products either from total J2.17 poly(A)⁺ RNA (lane 2) or from a receptor mRNA-enriched preparation (lane 3); in general, the ³⁵S-methionine-labelled proteins obtained from the two reactions are similar, although several species are more strongly labelled in lane 3. Three proteins specified by the enriched RNA were immunoprecipitated by receptor-specific monoclonal antibody (lanes 4, 5); two of these (the 94K and 79K species) were

readily observed among the total proteins synthesized from the enriched mRNA, while the third, a 91K protein, was detected only after precipitation. We were encouraged by this finding, because purified preparations of the 94K; rat liver glucocorticoid receptor often contain two discrete proteolytic degradation products which migrate as minor 91K protein and a more abundant 79K species⁵.

We calculated that 2% of the *in vitro* translation product from the immunologically purified RNA was receptor protein, and assumed that receptor mRNA was present at comparably high levels in that RNA preparation. Therefore, we constructed a cDNA library¹⁴, beginning with 0.4 μ g of enriched mRNA; 540 transformed bacterial colonies were screened by differential hybridization with labelled cDNA probes from enriched and unenriched RNA. Six independent clones were identified and shown to contain inserts of between 0.9 and 3.5 kb; all of the inserts cross-hybridize, and restriction mapping suggests that they represent overlapping clones from a single transcription unit (R.M. *et al.*, manuscript in preparation). In this study, we used a plasmid with a 2.6-kb insert (pRM16), shown by DNA sequencing to contain a segment of open reading frame and a putative poly(A)-addition signal (AATAAA) upstream of an 80-nucleotide poly(A) tract (S. Rusconi, unpublished data). Northern hybridizations revealed a ~6-kb transcript homologous to the pRM16 insert (see below) that had been selectively enriched ≥ 200 -fold by polysome immunoadsorption, corresponding closely with predictions based on the relative abundance of receptor translated from total poly(A)⁺ RNA ($\leq 0.01\%$) and from enriched RNA (2%).

Hybrid-selected translation¹⁵ was used to confirm that pRM16 contains sequences complementary to receptor mRNA. Enriched RNA was hybridized with pRM16, and as a control, with a β -tubulin cDNA plasmid, which were immobilized on separate nitrocellulose filters; RNA recovered from each filter was then translated *in vitro*. pRM16-selected mRNA yields translation products indistinguishable from intact receptor and its two apparent degradation products (Fig. 2); a comparison of lanes 1 and 3 of Fig. 2 reveals that the hybrid-selection reaction gave an overall recovery of about 10% of the intact receptor-coding RNA initially added, as expected for this procedure¹⁶.

The labelled pRM16 insert was used to probe Southern blots of genomic DNA from cultured rat cell lines of liver (J2.17) or fibroblast (XC) origin. Figure 3 shows that DNA from these two cell types yielded identical hybridization patterns after digestion with *Bam*HI (lanes 1, 4), *Eco*RI (lanes 2, 5) or *Hind*III (lanes 3, 6). Moreover, only two fragments were observed in

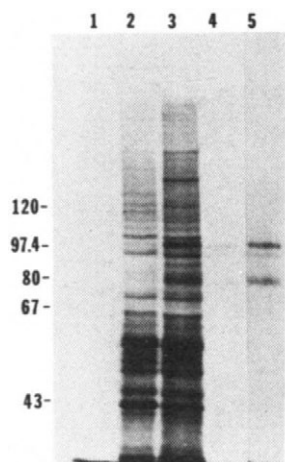


Fig. 1 Immunoprecipitation of ^{35}S -methionine-labelled receptor translated *in vitro*. Total translation products were from reactions programmed with no RNA (lane 1), 1 μg total poly(A) $^{+}$ RNA (lane 2), or $\sim 0.3 \mu\text{g}$ receptor-enriched RNA (lanes 3, 4) preparations. Products in lane 4 were immunoprecipitated with receptor-specific monoclonal antibody; lane 5, sixfold longer exposure of lane 4. No labelled proteins were recovered in a parallel precipitation with control ascites, or after precipitation of total poly(A) $^{+}$ RNA translation products with receptor antibodies (data not shown). Relative molecular mass standards ($\times 10^{-3}$), top to bottom: β -galactosidase, phosphorylase *b*, conalbumin and ovalbumin. Electrophoresis was in a 7% SDS-polyacrylamide gel; lanes 1 and 2 received 5% of the respective reaction products; lane 3, 15%; lane 4, 85%.

Methods: Polysome immunoadsorption was carried out essentially as described elsewhere^{28,29}; 2×10^9 J2.17 cells yielded about 1,000 A_{260} units of polysomes. A 1:1 mixture of a receptor-specific antiserum⁴ and a monoclonal antibody (IgG2a, no. 7; ref. 6) was purified with protein A-Sepharose to remove ribonucleases; 7 mg of the antibodies was incubated for 1–2 h at 4°C with 1,000 A_{260} units of polysomes in 30 ml of polysome buffer (25 mM Tris, pH 7.5, 5 mM MgCl_2 , 0.1% Nonidet P-40, 150 mM NaCl, 1 $\mu\text{g ml}^{-1}$ Trichodermin (Leo) and 0.5 mg ml^{-1} heparin), then passed twice through a 5-ml protein A-Sepharose column. The column was washed overnight with 200 ml polysome buffer followed by RNA elution with 20 ml of 25 mM Tris, pH 7.5, 20 mM EDTA, 0.5 mg ml^{-1} heparin; poly(A) $^{+}$ RNA was subsequently purified by oligo(dT)-cellulose chromatography, then ethanol precipitated in the presence of 10 $\mu\text{g ml}^{-1}$ bovine liver tRNA and 2 M ammonium acetate. About 2 μg of enriched RNA was recovered, as estimated from *in vitro* translation of known amounts of J2.17 total poly(A) $^{+}$ RNA. Reaction conditions for *in vitro* translation were exactly as described by the suppliers (Bethesda Research Laboratories or Promega Biotec) using 100 μCi ^{35}S -methionine (NEN 1,000 Ci mmol^{-1}) in 50 μl final volume. Immunoprecipitations³⁰ were done using monoclonal antibody IgG2a, no. 7 (ref. 6); control precipitations (not shown) used ascites fluid from mice injected with Sp2/0 myeloma cells, the parent line used in the hybridoma fusions⁶.

each digest, consistent with the notion that the receptor is encoded by a single-copy gene.

An extensive series of mouse lymphoma cell mutants have been isolated which fail to respond to glucocorticoids and carry certain distinct receptor defects: (1) receptor-deficient (r^{-}) mutants lack steroid-binding activity; (2) nuclear-transfer deficient (nt^{-}) lines produce a 94K M_r hormone-binding protein that interacts with reduced affinity with both nonspecific^{17,18} and specific¹⁹ DNA sequences; (3) increased nuclear-transfer (nt^+) mutants contain a hormone-binding species 40K M_r that lacks a major antigenic determinant²⁰, associates with non-specific DNA with increased affinity¹⁷ and binds specific DNA sequences with reduced affinity²¹. To determine whether the receptor alterations reflect lesions detectable at the RNA level, receptor transcripts from several such cell lines were compared by blot hybridization. These included two wild-type mouse lymphoma cell lines, WEH17 (ref. 22) and S49.1 (ref. 14); the

Fig. 2 Hybrid-selection of receptor RNA. Enriched RNA from $\sim 5 \times 10^8$ cells was hybridized to filters containing 10 μg of immobilized DNA from either a chicken β -tubulin cDNA plasmid (lane 2) or pRM16 (lane 3); these two lanes show immunoprecipitated *in vitro* translation products of the eluted RNA. The small amount of receptor detected in lane 2 probably reflects background hybridization due to relaxed stringency washes used in this hybrid-selection procedure³¹. Lane 1, immunoprecipitated *in vitro* translation products of enriched RNA ($\sim 5 \times 10^8$ cell equivalents) that was not subjected to hybrid-selection. Markers and gel electrophoresis conditions were as in Fig. 1.

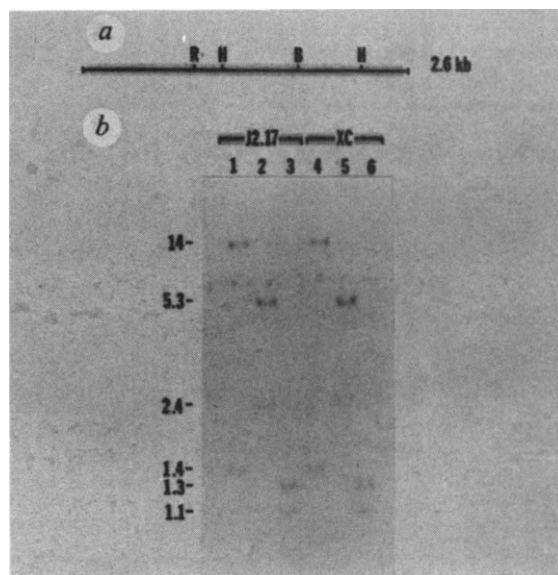
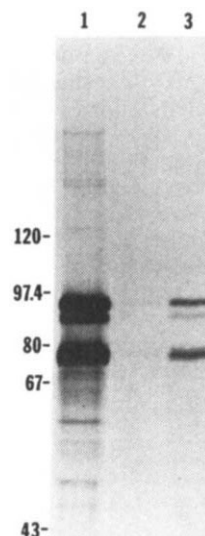


Fig. 3 Complexity of receptor sequences in genomic DNA from liver and fibroblast-derived rat cell lines. *a*, *Bam*HI (B), *Eco*RI (R) and *Hind*III (H) restriction sites within the 2.6-kb pRM16 inserts. Strand-specific cRNA probes used in Northern blot analysis (see Fig. 4) revealed that transcription proceeds from left to right. *b*, Southern blot using 20 μg of HTC (lanes 1–3) or XC (lanes 4–6) DNA was digested with *Bam*HI (lanes 1, 4), *Eco*RI (lanes 2, 5) or *Hind*III (lanes 3, 6), electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and probed with pRM16 using hybridization and wash conditions as previously described²⁷.

mutant lines, all derived from the latter parent, included an nt^{-} mutant, two nt^+ derivatives, and one r^{-} line. In addition, a rat HTC cell-derived r^{-} mutant, MSN5.3 (ref. 23), was tested.

Figure 4 shows a Northern blot in which equal amounts of total RNA from these cell lines were hybridized with pRM16 probe; interestingly, the probe detects rat and mouse sequences with apparently equal efficiency. In every case, a transcript of ~ 6 kb is observed, but the relative abundance of this transcript differs over a severalfold range among these cell lines; the same relative quantitative differences were seen in three independent RNA preparations. Thus, WEH17 contains two- to threefold more receptor RNA than S49.1 (lanes 3, 4), while the transcript in the nt^{-} mutant is indistinguishable from that in its parent (lanes 8, 4). In contrast, the nt^+ lines accumulate only about half as much of the 6-kb transcript as is found in the parent (lanes 4–6); similarly, the amount of the 6-kb transcript is reduced severalfold in both the mouse lymphoma r^{-} line and the rat

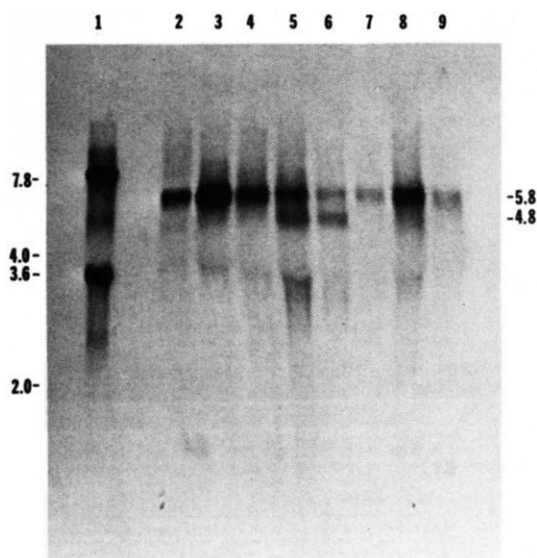


Fig. 4 Northern blot analysis of receptor RNA in wild-type and receptor mutant cell lines. Lanes 1-9 contain 20 µg of total RNA from rat HTC-derived (lanes 1, 2, 9) or mouse lymphoma (lanes 3-8) cell lines. The filter was hybridized with either nick-translated MTV DNA (lane 1) or pRM16 cRNA probe produced by SP6 polymerase³² (lanes 2-9). Cell lines and receptor phenotypes: M1.54, wild type (wt) (lanes 1, 2); WEH17, wt (lane 3); S49.1, wt (lane 4); S49.55R, ntⁱ (lane 5); S49.143R, ntⁱ (lane 6); S49.7R, r⁻ (lane 7); S49.22R, nt⁻ (lane 8); and MSN5.3, r⁻ (lane 9). Equivalent amounts of RNA were loaded in each lane, as assessed by hybridization with β-actin cDNA (not shown). The MTV transcripts (7.8 and 3.6 kb) and rRNAs (4.0 and 2.0 kb) were used as *M_r* to infer the sizes of the receptor transcripts; electrophoresis was in a 0.9% agarose-formaldehyde gel.

hepatoma r⁻ mutant relative to their respective parent lines (lanes 7 and 4 and lanes 9 and 2). In addition to these quantitative differences, both ntⁱ mutants contain an additional receptor transcript which migrates with an apparent size of ~5 kb and is present in these mutants at about the same relative abundance as the 6-kb transcript (lanes 5, 6). Southern blots of genomic DNAs from these wild-type and mutant cells were indistinguishable when monitored by pRM16 hybridization to *Eco*RI and *Hind*III restriction fragments (data not shown), suggesting that the mutant phenotypes do not reflect gross DNA rearrangements or deletions.

Westphal *et al.*²⁴ have recently analysed receptor protein in a variety of mouse lymphoma cells (including four studied here) using a combination of hormone binding and immunoreactivity measurements. They conclude that WEH17 produces relatively high levels of receptor from both chromosomal homologues, whereas S49, which appears to be functionally hemizygous^{23,25}, produces active receptor from one homologue while the other allele encodes a 94K protein that is immunoreactive but fails to bind steroid. Similarly, r⁻ mutants which lack hormone-binding activity nevertheless produce 20-50% as much 94K immunoreactive material as the wild-type parent. Our qualitative and quantitative characterizations of receptor transcripts correlate directly with those studies. Thus, we suggest that the 6-kb RNA present at reduced levels in r⁻ and ntⁱ mutants may encode the nonfunctional 94K protein, and that the 40K ntⁱ receptor may be encoded by the ntⁱ-specific 5-kb transcript. Finally, receptor mRNA in the nt⁻ mutant does not differ detectably from wild type, consistent with the bulk physical properties of this mutant protein¹⁸.

To our knowledge, this is the first reported isolation of sequences encoding a eukaryotic transcriptional regulatory protein whose activity is controlled by a defined physiologically important ligand. Moreover, the specific genomic sites of action have been determined at the nucleotide level for this protein¹², and its binding at these sites shown to regulate the novel process of

transcriptional enhancement^{11,26,27}. The elucidation of receptor gene organization and expression in wild-type and mutant cells will provide valuable information for studies on the mechanisms by which receptor regulates gene expression.

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- Anderson, J. N. in *Biological Regulation and Development* Vol. 3B (eds Goldberger, R. F. & Yamamoto, K. R.) 169-212 (Plenum, New York, 1984).
- Wrange, Ö., Carlstedt-Duke, J. & Gustafsson, J.-Å. *J. biol. Chem.* **254**, 9284-9290 (1979).
- Wrange, Ö., Okret, S., Radojicic, M., Carlstedt-Duke, J. & Gustafsson, J.-Å. *J. biol. Chem.* **259**, 4534-4541 (1984).
- Okret, S., Carlstedt-Duke, J., Wrange, Ö., Carlstrom, K. & Gustafsson, J.-I. *Biochim. biophys. Acta* **677**, 205 (1981).
- Westphal, H. M., Moldenhauer, G. & Beato, M. *EMBO J.* **1**, 1467-1471 (1982).
- Okret, S., Wikstrom, A.-C., Wrange, Ö., Andersson, B. & Gustafsson, J.-Å. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1609-1613 (1984).
- Payvar, F. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 6628-6632 (1981).
- Scheidereit, C., Geisse, S., Westphal, H. M. & Beato, M. *Nature* **304**, 749-752 (1983).
- Payvar, F. *et al. Cell* **35**, 381-392 (1983).
- Renkawitz, R., Shutz, G., von der Ahe, D. & Beato, M. *Cell* **37**, 503-510 (1984).
- Chandler, V. L., Maler, B. A. & Yamamoto, K. R. *Cell* **33**, 489-499 (1983).
- DeFranco, D., Wrange, Ö., Merryweather, J. & Yamamoto, K. R. in *Genome Rearrangement* (eds Herskowitz, I. & Simon, M.) (Liss, New York, in the press).
- Yamamoto, K. R., Stallcup, M. R., Ring, J. & Ringold, G. M. *Cold Spring Harb. Symp. quant. Biol.* **42**, 625-638 (1978).
- Okayama, H. & Berg, P. *Molec. cell. Biol.* **3**, 280-289 (1983).
- Miller, J. S., Paterson, B. M., Ricciardi, R. P., Cohen, L. & Roberts, B. E. *Meth. Enzym.* **101**, 650-674 (1983).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning* (Cold Spring Harbor Laboratory, New York, 1982).
- Yamamoto, K. R., Stampfer, M. R. & Tomkins, G. M. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3901-3905 (1974).
- Yamamoto, K. R., Gehring, U., Stampfer, M. R. & Sibley, C. *Rec. Prog. Horm. Res.* **32**, 3-32 (1976).
- Pfahl, M. *Cell* **31**, 475-482 (1982).
- Okret, S., *et al. Cancer Res.* **43**, 3127-3131 (1983).
- Payvar, F. & Wrange, Ö. *Nobel Symp.* **57**, 267-282 (1983).
- Bourgeois, S. & Newby, R. F. *Cell* **11**, 423-430 (1977).
- Grove, J. R., Dieckmann, B. S., Schroer, T. A. & Ringold, G. M. *Cell* **21**, 47-56 (1980).
- Westphal, H. M., Mugele, K., Beato, M. & Gehring, U. *EMBO J.* **3**, 1493-1498 (1984).
- Francke, U. & Gehring, U. *Cell* **22**, 657-664 (1980).
- Yamamoto, K. R. in *Transfer and Expression of Eukaryotic Genes* (eds Ginsberg, H. S. & Vogel, H. J.) 79-92 (Academic, New York, 1984).
- Zaret, K. S. & Yamamoto, K. R. *Cell* **38**, 29-38 (1982).
- Shapiro, S. Z. & Young, J. R. *J. biol. Chem.* **256**, 1495-1498 (1981).
- Korman, A. J., Knudsen, P. J., Kaufman, J. F. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1844-1848 (1982).
- Firestone, G. L., Payvar, F. & Yamamoto, K. R. *Nature* **300**, 221-225 (1982).
- Drubin, D. G., Caput, D. & Kirschner, M. W. *J. Cell Biol.* **98**, 1090-1097 (1984).
- Zinn, K., DiMaio, D. & Maniatis, Y. *Cell* **34**, 865-879 (1983).

Competition for cellular factors that activate metallothionein gene transcription

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Metallothioneins (MTs), small cysteine-rich proteins, bind to and are inducible by heavy metals such as zinc, cadmium and copper. Recent gene-transfer and mutagenesis experiments have elucidated cis-acting DNA sequences involved in this form of regulation^{1,2}, but nothing is known about the trans-acting factors that interact with the control sequences or how such interactions influence the

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rate of transcription. We report here the detection of cellular factors involved in the cadmium induction of the mouse *MT-I* gene by an *in vivo* competition assay. We show that at least one class of these cellular factors acts by a positive regulatory mechanism depending on the same region of the 5' flanking DNA required for maximal transcription.

In *Escherichia coli*, *lac* operator DNA at high copy number results in titration of *lac* repressor and constitutive transcription of the operon³. We have applied the same principle to mammalian cells, namely that if a regulatory factor is present in limited amounts it should be possible to titrate it by introducing sufficient control-sequence DNA into the cells. By determining whether competition leads to a decrease or increase in transcription, one can distinguish between positive regulation by an activator factor and negative regulation by a repressor. Furthermore, by varying the structure of the competitor DNA, it is possible to localize the interacting DNA sequences and to determine whether different genes are regulated by common or distinct factors. A similar approach has been used⁴ to detect cellular factors that interact with molecules containing viral enhancer sequences.

Cultured monkey kidney cells were transfected with a calcium phosphate co-precipitate of three different simian virus 40 (SV40)-based plasmid DNAs: (1) The indicator plasmid contains a mouse *MT-I-E. coli* chloramphenicol acetyltransferase (*MT-CAT*) fusion gene in which *MT* regulatory sequences drive the expression of bacterial enzyme coding sequences. This plasmid is used to monitor *MT* gene transcription determined by CAT enzyme measurements⁵. (2) The competitor plasmid, which is used to titrate out the cellular regulatory factors, also contains *MT* regulatory sequences, but these are not fused to the *CAT* gene. In most experiments we used *MT-gal* fusions so that the transcriptional activity of the competitor DNA could be measured in an independent transfection experiment¹. (3) The carrier plasmid, used to keep the total DNA concentration constant in each precipitation mixture, is identical to the competitor plasmid except that it lacks all of the *MT* regulatory sequences.

Following transfection, cells were incubated with or without cadmium, extracts prepared and CAT enzyme activities measured to determine the transcription levels of the indicator plasmid. Because all of the plasmids contain the origin of SV40 DNA replication (provided by an SV40-guanine phosphoribosyl transferase fusion gene in the indicator plasmid), and because SV40 large T antigen is provided by the competitor and carrier plasmids, these plasmids are capable of replicating in the transfected cells. From Southern blot analyses, we estimated intracellular copy numbers of 7×10^4 molecules of indicator DNA and 1.3×10^6 molecules of competitor plus carrier DNA per cell in these conditions (ref. 6, and data not shown). The plasmids also contain functional SV40 early and late promoters so that each assay includes a high concentration of SV40 control sequences (not regulated by heavy metals) as well as the inducible *MT* regulatory elements. This strategy was adapted to avoid artefacts caused by nonspecific competition for general transcription factors.

The predicted results of this experiment are shown in Fig. 1b. If transcription were negatively controlled by a limiting component, we would expect the competing DNA to titrate out the repressor molecule. Consequently, the basal level of transcription would increase while the induced level would remain constant. In contrast, if the gene were positively controlled by a limiting component, we would expect the competitor DNA to titrate out the activator molecule, resulting in a decrease in transcription in the presence of cadmium. Figure 2 shows the results of an actual competition experiment in which the indicator and competitor plasmids contained the same 2,000 base pairs (bp) of *MT* 5'-flanking sequences. The addition of increasing amounts of competitor DNA led to a sequential decrease in the expression of the indicator plasmid in the presence of cadmium but had little effect on the basal expression levels. This is consistent with titration of positively acting cellular

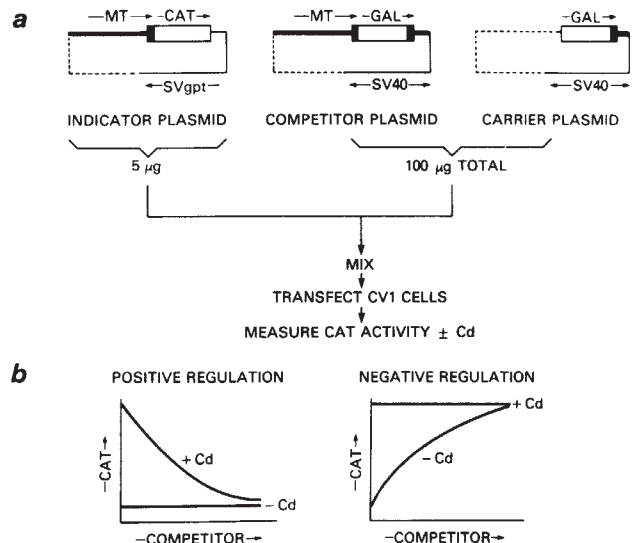


Fig. 1 Design and predicted results of the competition experiment. **a**, Cells were transfected with a mixture of indicator, competitor and carrier plasmids and incubated in the presence or absence of cadmium. Transcription from the *MT* promoter in the indicator plasmid was monitored by CAT enzyme measurements. Solid lines and bars indicate *MT* sequences, hollow bars indicate *E. coli* *CAT* and *gal* sequences, thin lines indicate SV40' sequences, and dotted lines indicate plasmid sequences. **b**, Predicted effects of adding increasing amounts of competitor plasmid on the expression of the *CAT* indicator plasmid. Positively regulated transcription would produce a decrease in the level of cadmium-induced expression (left panel), whereas negatively regulated transcription would produce an increase in the level of basal expression (right panel).

Methods: The indicator plasmid, *MT-CAT*, is a derivative of plasmid pSV2-cat (ref. 5) and contains: (1) 2,000 bp 5' flanking sequences and 68 bp 5' untranslated sequences from the mouse *MT-I* gene^{8,9}, inserted into the unique *Hind*III site of the vector through *Eco*RI-*Hind*III and *Bam*HI-*Hind*III adaptors; (2) the coding sequences for *E. coli* *CAT* followed by a fragment of SV40 DNA containing RNA splicing and polyadenylation sites; (3) an SVgpt transcription unit including the SV40 origin of DNA replication and early region promoter followed by the coding sequences for *E. coli* guanine phosphoribosyl transferase and SV40 splicing and polyadenylation sequences¹⁰, inserted at the *Bam*HI site of the vector; (4) *E. coli* plasmid pBR322 sequences including the origin of DNA replication and ampicillin resistance gene. The competitor plasmid, *MT-gal*, has been described previously^{1,8}. The carrier plasmid, previously designated as Δ galII (ref. 1), is identical to the competitor plasmid except that the mouse *MT-I* 5' flanking and 5' untranslated sequences have been deleted and replaced with a *Cla*I linker. Plasmids were constructed, characterized and propagated by standard methods¹¹.

factors (Fig. 1b, left panel). Quantitation of the CAT assays showed that the induction ratio for the indicator plasmid fell from 30-fold to 7-fold as the ratio of competitor to indicator plasmid was increased from 0 to 10. Moreover, a plot of the fraction of CAT activity remaining versus the ratio of competitor to competitor-plus-indicator DNA generated close to a straight line with a slope of -1. This is to be expected if the cellular factors interact with the indicator and competitor plasmids with the same affinity. These results were reproducible in multiple experiments ($\sigma = 20-30\%$).

To determine whether the titratable factors are specific for *MT* gene expression, we repeated the competition experiment using an α -globin-*MT* hybrid promoter that is efficiently transcribed but uses globin rather than *MT* upstream regulatory sequences (Fig. 3). This hybrid promoter contains *MT* gene sequences between positions -34 and +68 fused to upstream human α -globin gene sequences between positions -409 and -55 (Fig. 4). The *MT* portion of this construct includes the conserved 'TATA' sequence and the transcription initiation site—both thought to act as sites for interaction with general

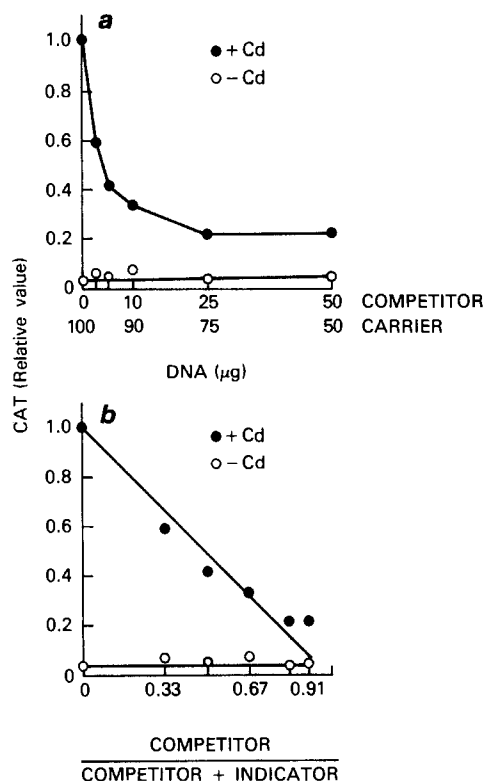


Fig. 2 Transcription is positively regulated. Cells were transfected with 5 μg *MT-CAT* indicator plasmid, 0–50 μg *MT-gal* competitor plasmid and sufficient carrier plasmid to give a total of 105 μg. The cells were incubated in the presence (●) or absence (○) of cadmium and CAT enzyme levels were determined. *a*, Relative CAT values as a function of the concentration of competitor plasmid; *b*, the same data as a function of the ratio of competitor to competitor plus indicator DNA. The relative CAT value is the ratio of CAT activity in the experimental extract to CAT activity in the extract from cells transfected without competitor DNA and induced with cadmium.

Methods: African green monkey kidney cells, line CV1, were seeded into 100-mm culture dishes and grown in Dulbecco's minimum essential medium with 10% fetal calf serum to $\sim 10^6$ cells per plate. Transfection was carried out by calcium phosphate precipitation using a minor modification of previously described methods^{1,5}. Each precipitation mixture contained 10 μg indicator plasmid and 200 μg of competitor plus carrier plasmid. Plasmid DNAs (10–200 μl) were added to 1 ml of 2×HEPES buffered saline (HBS) containing 0.274 M NaCl, 0.01 M KCl, 0.003 M Na₂HPO₄·7H₂O, 0.012 M dextrose, 0.042 M HEPES, pH adjusted to 7.1 with 10 M NaOH. The precipitate was formed by adding 1 ml 0.25 M CaCl₂ dropwise to the DNA/HBS solution with gentle mixing after each drop. The use of 0.003 M phosphate in the HBS solution gave finer precipitates and higher transfection efficiencies than the earlier method. It was also important to purify the plasmid DNA by two centrifugations in CsCl-ethidium bromide gradients. Duplicate plates of cells were incubated with 1 ml of DNA precipitate for 4 h, shocked for 3 min at 37°C with 15% glycerol in 1×HBS, incubated for 12 h in growth medium, then treated or not with 2 μM CdCl₂ for an additional 8 h. Cell extracts were prepared and CAT enzyme activity assayed as described previously⁵.

RNA polymerase II transcription factors—whereas the globin portion contains the upstream *CAT* sequence known to be an essential regulatory element for efficient globin gene transcription in monkey cells⁶. Transcription measurements, either by nuclease S₁ mapping of RNA or galactokinase assays on an α -*MT-gal* fusion gene, showed that this promoter is transcribed efficiently but is not inducible by cadmium (Fig. 4). However, when tested for competition against the *MT-CAT* indicator plasmid, no decrease in CAT activity was observed (Fig. 3*b*). In the converse of this experiment, we used the *MT-gal* plasmid to compete against an α -*MT-CAT* indicator plasmid and again observed no decrease in CAT activity. We also saw no competition by the *SV40* late and early promoters in an experiment

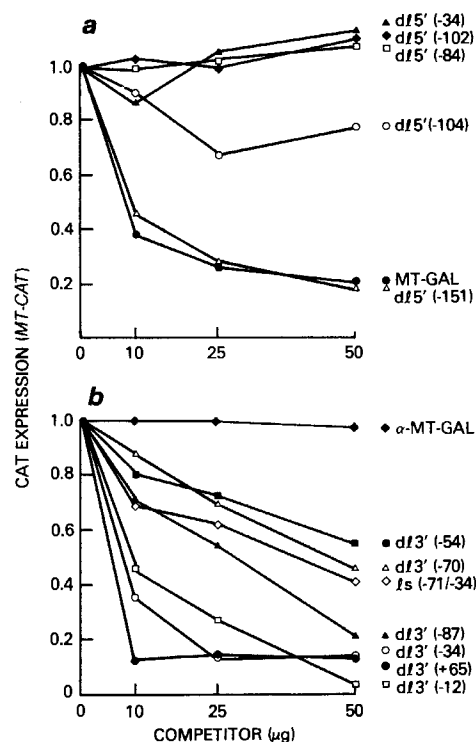


Fig. 3 Competition by deletion mutants and a hybrid promoter. Cells were transfected with 5 μg of *MT-CAT* indicator plasmid, 0–50 μg of *MT-gal* or the indicated construct as competitor, and sufficient carrier DNA to give a total of 105 μg. CAT enzyme levels were quantitated and plotted as described in Fig. 2 legend. Only the results for cadmium-induced cells are shown; basal levels were also measured but showed little or no change.

Methods: In the 5' deletion mutants (constructed as described in ref. 1), d15' (*n*), all the *MT* 5' flanking sequences between positions –2,000 and *n* are replaced by plasmid DNA and the *MT* gene is fused to *gal* at position +68. In the internal deletion, Is (–71/–34), the *MT* sequences between positions –71 and –34 are replaced by a *Clal* linker and the construct is fused to *gal*. In the 3' deletions, d13' (*m*), the *MT* gene and flanking sequences between positions +2,000 and *m* are replaced by plasmid DNA. The 3' deletions to +65 and –15 were also fused to *gal* to measure transcription levels in independent experiments. The α -*MT-gal* construct is identical to the standard *MT-gal* competitor plasmid except that the *MT* 5' flanking sequences between positions –34 and –2,000 are replaced by a fragment of the human α -globin gene between positions –55 and –409.

using pBR322-SV40 as competitor and pBR322 as carrier (data not shown). We conclude that the titratable cellular factors are specific for *MT* gene expression and do not represent general RNA polymerase II transcription factors.

To determine the relationship between the DNA sequences responsible for titration of the cellular factors and the transcriptional control sequences of the mouse *MT-I* gene, we performed competition assays on a series of 5', 3' and internal deletion mutants. Figure 3 shows the competition curves and Fig. 4 summarizes the results obtained from previous transcription assays⁷ and the present competition experiments. The following results were obtained: (1) A 5' deletion of all the upstream flanking DNA to position –151 competed as well as the starting gene with 2,000 base pairs (bp) of 5' flanking DNA. This mutant was inducible by cadmium and was transcribed $\sim 40\%$ as efficiently as the starting gene. (2) A deletion to position –104 showed very little competition and further deletions to –102 or –84 gave no detectable competition in our assay conditions. (The maximum amount of competitor DNA tested in these experiments was 50 μg, 10-fold greater than the amount of indicator plasmid; higher concentrations of competitor DNA gave irreproducible results.) These mutants were inducible by cadmium but had basal and induced transcription levels only 1–2% of that of the starting gene. A further deletion to position

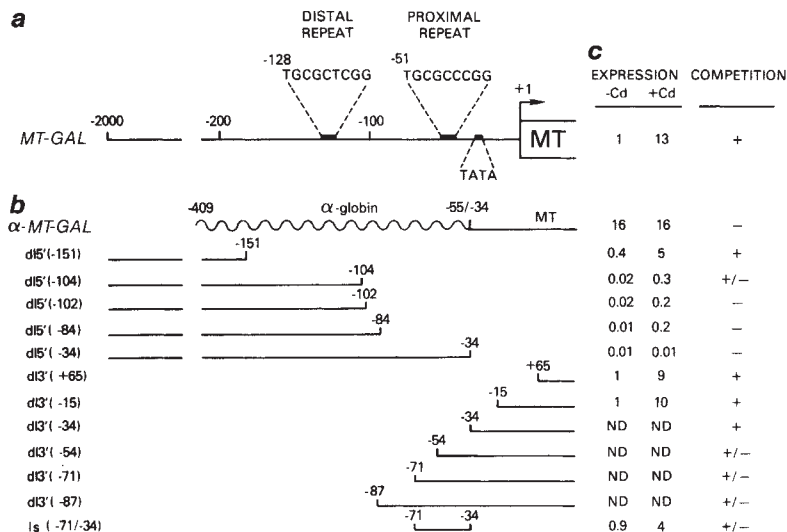


Fig. 4 Relationship between competing DNA sequences and transcriptional control elements. **a**, The structure of the mouse *MT-I* gene 5' flanking region showing the positions and sequences of the repeated nonanucleotide and the conserved TATA box. **b**, The structures of the various constructs used in competition experiments. **c**, Summary of transcription and competition data. The basal and induced transcription levels of these mutants were determined by galactokinase measurements and nuclease S_1 mapping of RNA¹; the basal level for the intact promoter is arbitrarily set at 1. The competition curves for these mutants are shown in Fig. 3.

-34 also failed to compete and was not inducible by cadmium. (3) The 3' deletions of the 5' untranslated and flanking sequences to positions +65 or -15 competed as well as the starting gene and were transcribed normally. A deletion to -34, which lacks the conserved TATA box region, also competed efficiently. (4) Further 3' deletions to positions -54, -71 or -87 and an internal deletion between positions -34 and -71 showed partial competition. In each case, two- to eightfold more deletion mutant than wild-type *MT-GAL* DNA was required to obtain a given level of competition. Transcription measurements on the internal deletion mutant showed that it had a normal level of basal expression but a reduced level of expression in the presence of cadmium.

These results demonstrate a strong correlation between the competing DNA sequences and the previously defined distal and proximal heavy metal control regions of the mouse *MT-I* gene¹. The distal control region, between positions -151 and -78, is essential for both efficient transcription and competition. The proximal region, between positions -78 and -15, is a much weaker transcriptional element and shows no competition in our experimental conditions. The failure of this region to compete is interesting because it gives a normal induction ratio for transcription. Thus it appears that titration of the cellular factor depends on the ability of a DNA sequence to promote efficient transcription in the presence of cadmium, not on the ability to be induced over the basal level. We observed also that the proximal region enhances transcription and competition by factors of two- to eightfold when present in conjunction with the distal region. This may be caused by two regions acting cooperatively or synergistically. For example, weak binding of factors in the proximal region could facilitate stronger binding in the distal region either by protein-protein contacts or by altering the secondary or tertiary structure of the DNA helix.

The distal and proximal control regions contain a duplicated nonanucleotide, present once in the proximal region and once in the distal region, that is conserved in human and rat *MT* genes and that has been implicated in heavy metal regulation^{1,2} (see Fig. 4). These repeats flank a G-rich sequence that may play a role in determining the efficiency of expression. At present we do not know which, if either, of these sequences is responsible for competition. However, it is clear that the nonanucleotide alone is insufficient as 5' deletion mutants retaining one copy of this sequence fail to compete. It is also known that the overall efficiency of *MT-I* gene transcription is affected by the sequences at the TATA box, the cap site and the far upstream region^{1,2}. We show here that these sequences are not responsible for titration of the positively acting cellular components.

We observed specific competition for the cellular regulatory factors only at input DNA concentrations in the range of 5–50 μ g per 10^6 cells, giving intracellular copy numbers of more than 10^6 total plasmids per cell following transfection and replication.

However, because the fraction of the molecules actually available for competition is unknown, we cannot calculate the precise concentration of the regulatory factors. Nevertheless, the high DNA concentrations required, together with the observation that chromosomally amplified *MT* genes are appropriately induced by heavy metals⁷, suggests that there are many active regulatory molecules per cell. We have previously shown that the mouse *MT-I* gene is regulated appropriately when introduced into cells on a replicating viral *SV40* vector⁸, and we observe here good induction ratios in cells transfected with limited quantities (5 μ g per 10^6 cells) of *SV40* plasmid vectors. In these experimental conditions the *MT* control sequences are apparently not present in sufficient quantities to titrate the cellular factors. Thus, in using the competition assay to study the regulation of other genes, it may be necessary to test a wide range of input DNA concentrations to obtain saturation.

The mouse *MT-I* gene shows a substantial level of transcription even when heavy metals are not added to the culture medium. One possible explanation for this is that the cells always contain some intracellular metal and hence some active *MT* transcriptional factors. For example, zinc and copper are inducers of *MT* synthesis which are also required for cell growth and are present in fetal calf serum in substantial concentrations (A. Leone and D.H.H., unpublished results). Alternatively, the *MT* control region may have intrinsic promoter activity even in the absence of active transcriptional factors. The lack of competition for basal transcription at the DNA concentrations tested argues for the second of these models.

The *in vivo* competition assay described here differs in several important aspects from the more traditional transcription assays and may be useful for studying the regulation of other cloned eukaryotic genes. First, the assay allows the formal differentiation between positive and negative transcriptional regulation. Second, because competition can be measured independently of transcription, specific control sequences can be distinguished from general promoter sequences even if they overlap. Finally, the assay can be used to determine whether different genes are regulated by distinct or common cellular factors. We observed no competition between *MT* and *SV40* or *MT* and globin promoters, indicating that they depend on different factors from those described in ref. 4.

The molecular nature of the *MT* regulatory factors is unknown. The present work suggests that at least one class of factors is present in multiple copies and preferentially interacts with the *MT* gene control sequences in metal-stimulated cells.

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1. Carter, A. D. *et al.* *Proc. natn. Acad. Sci. U.S.A.* (in the press).
2. Karin, M. *et al.* *Nature* **308**, 513-519 (1984).
3. Miller, J. H. & Reznikoff, W. S. *The Operon* (Cold Spring Harbor Laboratory, New York, 1980).
4. Scholer, H. R. & Gruss, P. *Cell* **36**, 403-411 (1984).
5. Gorman, C. M., Moffat, L. F. & Howard, B. H. *Molec. cell. Biol.* **2**, 1044-1051 (1982).
6. Mellon, P., Parker, V., Gluzman, Y. & Maniatis, T. *Cell* **27**, 279-288 (1981).
7. Beach, L. R. & Palmiter, R. D. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2110-2114 (1981).
8. Hamer, D. H. & Walling, M. J. *J. molec. appl. Genet.* **1**, 273-288 (1982).
9. Glanville, N., Durnam, D. M. & Palmiter, R. D. *Nature* **292**, 267-269 (1981).
10. Mulligan, R. C. & Berg, P. *Science* **209**, 1422-1427 (1980).
11. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning* (Cold Spring Harbor Laboratory, New York, 1982).

Virus- and cell-encoded tyrosine protein kinases inactivate DNA topoisomerases *in vitro*

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Tyrosine protein kinase activity is associated with at least eight different retrovirus-encoded *onc* gene products¹ and with cell receptors for epidermal growth factor, platelet-derived growth factor, tumour growth factor and insulin²⁻⁵. Both the *onc* kinases and the growth factor receptors are membrane proteins whose enzymatic activity has been implicated in stimulation of growth. However, the mechanism by which a signal passes from the plasma membrane to the nucleus to initiate growth remains unknown. As DNA topoisomerases catalyse the interconversion of topological isomers of DNA and hence affect DNA replication, transcription and recombination⁶⁻⁸, they may be involved also in stimulation of growth. Several DNA topoisomerases have been shown to form a covalent complex with DNA via a phosphotyrosine linkage^{9,10}. The DNA-protein complex is postulated to be an intermediate in breaking and rejoining of DNA. The aim of the present study was to determine whether tyrosine protein kinases modulate the activity of topoisomerases by phosphorylating the tyrosine residue involved in DNA binding. We report that incubation of *Escherichia coli* and calf thymus type I DNA topoisomerases with the Rous sarcoma virus transforming gene product, pp60^{src}, and TPK75, a tyrosine protein kinase purified from normal rat liver^{11,12}, results in a 10-fold loss of topoisomerase activity.

That an essential tyrosine residue in the active site of topoisomerases acts as a nucleophile is implied by the covalent linkage to DNA via a phosphotyrosine bond^{9,10}. Indeed, chemical modification of the tyrosine residues in *Micrococcus luteus* topoisomerase I and DNA gyrase with tetranitromethane leads to inactivation of the enzyme¹³.

Figure 1 shows the phosphorylation *in vitro* by pp60^{src} of *E. coli* type I DNA topoisomerase (lane 2), *M. luteus* DNA gyrase (lane 3) and calf thymus type I (lane 4) and type II (lane 5) DNA topoisomerases, and the phosphorylation by TPK75 of calf thymus type I (lane 6) and *E. coli* type I (lane 7) DNA topoisomerases. None of the topoisomerase preparations has endogenous protein kinase activity in the condition used here. Subunit A of DNA gyrase (Fig. 1, lane 3), which exhibits the DNA breaking-rejoining activity, was phosphorylated to a much greater extent than subunit B, which possesses the ATPase activity. Examination of the pattern obtained by SDS-gel electrophoresis of the topoisomerases revealed that *E. coli* DNA topoisomerase I and DNA gyrase are homogeneous. The only band visible by staining in the calf thymus topoisomerase I preparation has a relative molecular mass (*M_r*) of 85,000 (85K) while that of the calf thymus topoisomerase II is 98K. Bands of these relative molecular masses were indeed phosphorylated by the protein kinases. To determine which amino acids in the topoisomerases were phosphorylated, the topoisomerases were eluted from the SDS gel and subjected to acid hydrolysis, then

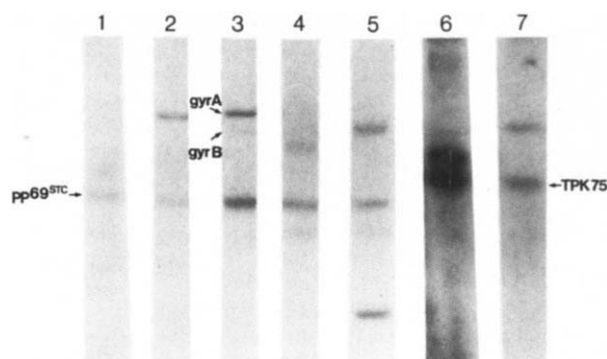


Fig. 1 Autoradiogram of a SDS-polyacrylamide gel showing the *in vitro* phosphorylation of DNA topoisomerases by tyrosine protein kinases. Lane 1, pp60^{src}; 2, pp60^{src} + *E. coli* type I DNA topoisomerase; 3, pp60^{src} + *M. luteus* DNA gyrase; 4, pp60^{src} + calf thymus type I DNA topoisomerase; 5, pp60^{src} + calf thymus type II DNA topoisomerase; 6, TPK75 + calf thymus type I DNA topoisomerase; 7, TPK75 + *E. coli* type I DNA topoisomerase. In each lane, the topoisomerase corresponds to the band of highest molecular weight. The calf thymus DNA topoisomerases used were slightly degraded. Some smaller polypeptides were phosphorylated together with the major ones.

Methods: Each reaction mixture (10-25 μ l) contained 2-5 μ l of either purified pp60^{src} (0.01 unit in 10 μ l, 1 unit being the amount required to transfer 1 pmol of phosphate per min at 30 °C into [Val³]angiotensin peptide⁶) from Rous sarcoma virus-transformed rat fibroblast cells, or partially purified TPK75 from rat liver (both preparations were free of cyclic AMP-dependent kinase activity), 10 mM Tris pH 6.8, 3 mM MnCl₂, 0.05% 2-mercaptoethanol, 10 μ Ci [γ -³²P]ATP (3,000 Ci mmol⁻¹) and 1 μ g DNA topoisomerase. After incubation at 30 °C for 30 min, the proteins were precipitated with trichloroacetic acid and analysed by electrophoresis in a 6% SDS-polyacrylamide gel. *E. coli* type I DNA topoisomerase was given by Dr J. C. Wang, and calf thymus type I and type II DNA topoisomerases by Dr L. F. Liu. A sample of calf thymus type I DNA topoisomerase was also purchased from BRL, together with *M. luteus* DNA gyrase.

analysed by thin-layer electrophoresis and chromatography⁹; in all cases phosphorylated residues were found to be tyrosine (data not shown).

We next examined the effect of phosphorylation on topoisomerase activity. After incubation of *E. coli* type I DNA topoisomerase with pp60^{src} and a millimolar amount of ATP, the enzyme was diluted and assayed for its ability to relax supercoiled pUC8 plasmid DNA. Figure 2 (lanes 1, 3) shows that phosphorylation of the enzyme resulted in the loss of >90% of *E. coli* type I DNA topoisomerase activity; a similar loss of enzyme activity was seen following incubation of calf thymus type I DNA topoisomerase with TPK75 (Fig. 2, compare lanes 7 and 8 with 10 and 11). In separate experiments, we demonstrated that the tyrosine protein kinases had no effect on DNA superhelicity and that incubation of the type I topoisomerases with cyclic AMP-dependent protein kinases from beef heart and rabbit muscle (Sigma) did not result in phosphorylation of the topoisomerases or loss of topoisomerase activity.

We attempted to determine the effect of phosphorylation on the type II topoisomerase activities, but these were much less stable than the type I topoisomerase activities in conditions that were optimal for protein kinase activity. Therefore, even in the control samples without the protein kinase, the type II topoisomerase activities were lost rapidly, making it difficult to evaluate the effect of phosphorylation.

To the best of our knowledge, this represents the first demonstration of modulation of an enzymatic activity other than that of protein kinase via *in vitro* phosphorylation of tyrosine residues of the enzyme. The biological significance of this reaction is presently unknown. Loss of bacterial type I DNA topoisomerase activity *in vivo* results in more highly negatively supercoiled DNA¹⁴. Recent studies on chromatin assembly in *Xenopus* oocytes indicate that in eukaryotes, DNA supercoiling

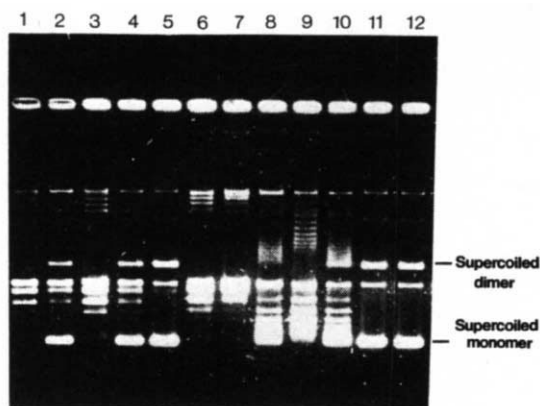


Fig. 2 Agarose gel electrophoresis of plasmid DNA, showing the effect of tyrosine protein kinases on the activity of type I DNA topoisomerases. The following were added to samples of supercoiled DNA: lane 1, 20 ng and lane 2, 2 ng of *E. coli* DNA topoisomerase I incubated without pp60^{src}; lanes 3–5, 200, 20 and 2 ng respectively of *E. coli* DNA topoisomerase I incubated with pp60^{src}; lanes 6–8, 1, 0.1 and 0.01 units respectively of calf thymus DNA topoisomerase I incubated without TPK75; lanes 9–11, 1, 0.1 and 0.01 unit respectively of calf thymus DNA topoisomerase I incubated with TPK75; lane 12, no topoisomerase.

Methods: Topoisomerase and protein kinase were incubated for 60 min in conditions described in Fig. 1 legend, except that 1 mM ATP was used instead of [γ -³²P]ATP. The topoisomerase was then diluted and assayed. The reaction mixture for *E. coli* DNA topoisomerase I contained 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 100 μ g ml⁻¹ gelatin, 6 mM MgCl₂ and 0.5 μ g of supercoiled pUC8 plasmid DNA. The reaction mixture for calf thymus DNA topoisomerase I contained 150 mM NaCl, 20 mM Tris-HCl pH 8, 100 μ g ml⁻¹ gelatin, 1 mM EDTA and 0.5 μ g of supercoiled plasmid DNA. After incubation at 37 °C for 30 min, the reaction was stopped by adding 1% SDS, and the mixture analysed by electrophoresis on a 0.8% agarose gel.

and chromatin assembly are probably coupled *in vivo*¹⁵. The degree of DNA supercoiling in transcriptionally active chromatin is probably controlled by balancing the relaxing activity of DNA topoisomerase I and the supercoiling activity of a 'gyrase' consisting of DNA topoisomerase II coupled with ATP and unknown factor(s). A correlation between the proliferation state of eukaryotic cells and DNA superhelicity has been observed by several investigators^{16–18}. When the rate of cell growth is high, DNA is more negatively supercoiled, and vice versa^{16–18}; there is no clear explanation for this alteration of supercoiling. Postsynthetic modification of topoisomerases may represent one possible mechanism of regulating cellular proliferation. The products of retroviral oncogenes, including several membrane-associated tyrosine protein kinases¹⁹, and membrane-associated growth factor receptor tyrosine protein kinases (such as that for epidermal growth factor) share the ability to stimulate growth, possibly through common mechanisms. Such stimulation of growth must involve activation of replicative processes and increased levels of transcription and translation. It will be of interest to determine whether retroviral- or cellular-encoded protein kinases affect topoisomerases either by direct interaction or through another tyrosine protein kinase that shuttles from the plasma membrane to the nucleus, transmitting a growth signal. For example, it is known that the binding of epidermal growth factor to the plasma membrane results in the internalization of a portion of the receptor molecule^{20,21}.

We propose that the receptor kinase might affect growth processes by either migrating to the nucleus and directly phosphorylating a topoisomerase, or by phosphorylating another kinase which in turn passes to the nucleus and phosphorylates a topoisomerase. The same possibilities exist for growth signal transmission by plasma membrane-associated viral tyrosine kinases. However, the activity of such kinases should be independent of an external growth signal. Although there exist no data indicating that viral tyrosine kinases are internalized,

experiments from our laboratory and elsewhere have shown that proteolytic fragments of pp60^{src} contain kinase activity¹⁹. In addition to phosphorylation by tyrosine protein kinases, it has been shown previously that DNA topoisomerases from various sources can be modified *in vitro* by phosphorylation of a serine residue²², or by poly(ADP) ribosylation²³. It remains to be determined which, if any, of these reactions is important *in vivo*.

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1. Bishop, J. M. & Varmus, H. In *Molecular Biology of Tumor Viruses: RNA Tumor Virus* 2nd edn (eds Weiss, R. Teich, N., Varmus, H. & Coffin, J.) 999–1108 (Cold Spring Harbor Laboratory, New York, 1982).
2. Ushiro, H. & Cohen, S. *J. biol. Chem.* **255**, 8363–8365 (1980).
3. Reynolds, F. H. Jr, Todaro, G. J., Fryling, C. & Stephenson, J. R. *Nature* **292**, 259–262 (1981).
4. Ek, B., Westermark, B., Wasteson, A. & Heldin, C.-H. *Nature* **295**, 419–420 (1982).
5. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M. & Kahn, C. R. *Nature* **295**, 667–669 (1982).
6. Cozzarelli, N. R. *Science* **207**, 953–960 (1980).
7. Gellert, M. A. *Rev. Biochem.* **50**, 879–910 (1981).
8. Wang, J. C. *The Enzymes* **14**, 332–344 (1981).
9. Tse, Y.-C., Kirkegaard, K. & Wang, J. C. *J. biol. Chem.* **255**, 5560–5565 (1980).
10. Champoux, J. J. *J. biol. Chem.* **256**, 4805–4809 (1981).
11. Wong, T. W. & Goldberg, A. R. *J. biol. Chem.* **258**, 1022–1025 (1983).
12. Wong, T. W. & Goldberg, A. R. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2529–2533 (1983).
13. Klevan, L. & Tse, Y.-C. *Biochim. biophys. Acta* **745**, 175–180 (1983).
14. Pruss, G. J., Manes, S. H. & Drlaca, K. *Cell* **31**, 35–42 (1982).
15. Ryjoi, M. & Worcel, A. *Cell* **37**, 21–32 (1984).
16. Luchnik, A. N., Bakayev, V. V. & Glaser, V. M. *Cold Spring Harb. Symp. quant. Biol.* **46**, 793–799 (1982).
17. Glaser, V. M. & Luchnik, A. N. *Exp. Cell Res.* **139**, 249–255 (1982).
18. Huleihel, M. & Aboud, M. *J. Virol.* **48**, 120–126 (1983).
19. Krueger, J. G., Garber, E. A. & Goldberg, A. R. *Curr. Topics Microbiol. Immun.* **167**, 51–124 (1983).
20. Haigler, H. T., Ash, J. F., Singer, J. J. & Cohen, S. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3317–3321 (1978).
21. Haigler, H. T., McKanna, J. A. & Cohen, S. *J. Cell Biol.* **81**, 382–395 (1979).
22. Durban, E., Mills, J. S., Roll, D. & Busch, H. *Biochem. biophys. Res. Commun.* **111**, 897–905 (1983).
23. Jongstra-Bilen, J., Ittel, M.-E., Niedergang, C., Vosberg, H.-P. & Mundel, P. *Eur. J. Biochem.* **136**, 391–396 (1983).

Errata

Unusual abundance of glyceraldehyde 3-phosphate dehydrogenase pseudogenes in vertebrate genomes

M. Piechaczyk, J. M. Blanchard, S. Riaad-el Sabouty, C. Dani, L. Marty & P. Jeanteur*

Nature **312**, 469–471 (1984)

IN the issue of 29 November the above letter appeared with an incorrect title.

Modelling the global climate response to orbital forcing and atmospheric carbon dioxide changes

N. G. Pias & N. J. Shackleton

Nature **310**, 757–759 (1984)

THE legend to Fig. 1 was incorrect and should read:

Fig. 1 Linear variance spectra for *a*, solar insolation at 65° N in June; *b*, the benthic oxygen isotope record from core V19-30; *c*, the simulated ice volume from the simple ice sheet model of Imbrie and Imbrie⁸; and *d*, for the time derivative of the benthic oxygen isotope record from core V19-30. All spectral calculations are from 326 data points spanning the last 340 kyr. Bandwidth shown is for spectral calculations from 120 points of the sample auto-covariance functions. Important periods associated with orbital parameters are labelled.

Physics at the frontier

Abdus Salam

Superforce: The Search for a Grand Unified Theory of Nature.

By Paul Davies.

Simon & Schuster/Heinemann: 1984. Pp.255. \$16.95, £12.95.

Like many compelling images, it may turn out to be a mirage, but for the first time in the history of science we can form a conception of what a complete scientific theory of the world will look like. Paul Davies in *Superforce*.

THERE is no question but that there has been a transformation, during the past decade, in our understanding of the inanimate physical Universe — a “quantum” jump of the type which from time to time takes place in the history of science. It is this leap in knowledge that Paul Davies sets out to describe in his latest book.

Some eleven years ago, around 1973, the situation was this: we had just begun to believe that all matter in the Universe was made from quarks and leptons (electrons, muons and neutrinos). We believed then that there were four distinct forces controlling all interactions of matter — the universal force of gravity; the force of electromagnetism, acting between electrically charged quarks and leptons; and the two nuclear forces, the strong nuclear force, between quarks, and the weak nuclear force, between left-spinning quarks and leptons. So far as the cosmological structure of the Universe was concerned, observations supported a “standard” model of an expanding Universe; this model stipulated a Universe beginning with a mysterious big bang. We could trace the orderly evolution of the Universe — the formation and emergence of the familiar nuclei, atoms, stars and galaxies — with an epoch starting one-hundredth of a second after time began. But the earlier history was unknown.

The transformation which has taken place since that time lies primarily in the understanding of the nature of the four forces and a comprehension of the earliest history of the Universe. We believe today that the primary agents responsible for the four fundamental forces are the so-called “gauge particles”. The prototype gauge particle is the quantum of light — the photon — which is the “gauge messenger” for the force of electromagnetism. During the past ten years, there has been experimental confirmation of the existence of gauge particles responsible for the strong nuclear force between quarks, and, even more dramatically, the discovery — in 1983 — of the gauge particles of the weak nuclear forces, the so-called Ws and the Zs.

Even more important than the unveiling of these objects has been the experimental confirmation of a theory, first proposed in 1967, that the two seemingly distinct forces — electromagnetism and the weak nuclear

force — share a common origin and are indeed aspects of a single unified force, the electroweak force. The clearest manifestation of this unification would need temperatures of the order of 10^{16} K or the laboratory provision of accelerator energies in excess of 100 GeV. Such an accelerator was built at CERN in Geneva in 1982 and the Ws and the Zs were duly created last year, with their theoretically predicted masses, in proton-anti-proton collisions.

So far as the history of the early Universe is concerned, we now believe that there was an epoch — lasting up to 10^{-12} s after time began — when temperatures in excess of 10^{16} K did obtain. During this epoch the Ws and the Zs shared with the photon a state of masslessness. A phase transition took place when temperatures fell below 10^{16} K, concomitant with the expansion of the Universe and its cooling. It was this phase transformation which was responsible for producing the distinction between electromagnetism and the weak nuclear force which we observe today. Before this epoch there was no distinction. With electroweak unification, we have thus pushed back the known life span of the early Universe by ten orders of magnitude.

So much for what is established and non-speculative in the current thinking. With the success of these ideas however, the question arose: are the other two forces, the strong nuclear and the gravitational, also united with the electroweak? Are we dealing in the end with just one force? Twelve years ago, speculation began on this subject. As regards the unification of the strong nuclear force with the electroweak, an attractive version of such a “grand” unification suggested that perhaps another phase transformation did take place some 10^{-31} s after time began. This would extend our knowledge of the early Universe by another 20 orders of magnitude in time.

One hard consequence of such a second unification would be the prediction of the instability of the proton, which in this picture would have a half-life of the order of 10^{38} s. Experimental confirmation of this decay has been sought but not yet conclusively demonstrated. Yet, a phase transition of the type implied in this “grand unification” is not to be abandoned lightly, for this gives the best explanation of the otherwise mysterious predominance of matter over anti-matter as observed in the Universe today. Thus the search for proton decay is still on, and will remain so.

In a finely written book, Paul Davies

takes us through all of these developments. He starts *ab initio*, assuming no prior knowledge of quantum theory or relativity theory, and gives a lucid exposition of these two earlier revolutions in our thinking. He then discusses the unification ideas I have mentioned above, and the relevance of the phase transitions in the early history of the expanding Universe, before moving onto the ideas currently occupying the centre of the theoretical stage which attempt to include the fourth force — gravity — in the scenario. Naturally, these ideas are highly speculative in nature. Among them is the postulate of a new type of symmetry — the so-called supersymmetry — combined with a postulate of extra dimensions of space, in what is known as the Kaluza-Klein framework. The synthesis of these two ideas gives rise to a discussion of the “superforce” of the book’s title, a concept which draws together supermatter (quarks, leptons, gauge particles and their superpartners) into one unified whole with the force of supergravity (described by gravitons and their speculated superpartners, the gravitinos). The theory is not yet in any final shape, but it possesses a beguiling aesthetic attractiveness, particularly when formulated in 11 dimensions.

Davies also gives an account of the associated phase transitions in the earliest history of the Universe, including the recent ideas of its rapid inflationary expansion and the possible origin of the Universe as a “quantum fluctuation” of the vacuum, out of “nothingness”.

This is new, heady stuff, professionally and accurately rendered. However, if I have one criticism of this eminently readable book, it is this. In the final part, one does not get the impression that the author is writing about material which is so highly speculative. The criticism applies also to a comment in the penultimate chapter:

The idea that non-causal, holistic order exists in the universe by no means originated with modern physics. Astrology, for example, is an attempt to discern a cosmic order in which the affairs of human beings are reflected in the organization of the heavens. The psychoanalyst Carl Jung and the quantum physicist Wolfgang Pauli proposed a non-causal connecting principle which they called synchronicity. They compiled evidence for a sort of pervasive order in which apparently independent events occur in conjunction in a meaningful way. Typical of such events are documented instances of extraordinary coincidences, well beyond the expectations of chance.

There are insufficient cautionary warnings to the reader that such thinking — about astrology and “coincidences” — is not the staple of physics. But this apart, unquestionably, Paul Davies has established himself as one of the most felicitous writers on physics at the frontier. □

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Outside eye on the hominid beat

Leonard Krishtalka

Human Evolution: An Illustrated Introduction.

By Roger Lewin.

Blackwell Scientific/W.H. Freeman: 1984. Pp.104. Hbk \$22.36; pbk £6.80, \$14.95.

PALAEoANTHROPOLOGY is familiar terrain to Roger Lewin. With Richard Leakey, he wrote *Origins* and *People of the Lake*, and he regularly covers the hominid beat, among other subjects, for *Science*. Not surprisingly, *Human Evolution: An Illustrated Introduction* is a testament to Lewin's expertise at science journalism. The writing is deft, the science lucid and the attitude no-nonsense.

All of the main characters in palaeo-anthropology are here — *Aegyptopithecus*, *Ramapithecus*, *Sivapithecus*, the australopithecines and *Homo* — along with the basic plot: the origin and evolution of hominids, bipedalism, large brain size, tool technology and social structures. Lewin ties the fossil record, morphology and, as appropriate, the archaeology of the various taxa to reconstructions of their evolutionary relationships, palaeoecology and cultural adaptations. This core information occupies only 70 or so of the 99 pages. The remainder are devoted to first principles and to the auxiliary disciplines that impinge on hominid palaeontology: highlights of the evolution of life on Earth; primate origins, diversification and disper-

sal; Neogene climates and geology; evolutionary theory; molecular-based phylogenies; taphonomy; ecological theory and more. Lewin also steers a clear and even-handed course through such contentious issues as phyletic gradualism versus punctuated equilibrium, molecular versus palaeontological divergence clocks, the status of *Australopithecus afarensis*, socio-biology and so on.

This is not a personal work. Lewin is the journalist throughout, reporting the consensus and disagreements, and deferring in most instances to the conclusions of the principal investigators in each field. And the presentation is balanced: comprehensive yet concise, not overly technical for the general reader yet meaty enough for most students of human origins. The index is detailed and the selected bibliography adequate.

My one quibble concerns the illustrations. They complement and supplement the text, but curiously are not numbered; nowhere are they tied to the written material. Although most of the figures appear near the appropriate text, the usual in-text references to figure so and so would have been helpful. Also, a few of the figure captions could be more explanatory, especially for those illustrations taken from technical research papers.

Over the past few years palaeoanthropology has matured into a vigorous multidisciplinary science. Lewin's *Human Evolution* is a concise primer on the subject which a wide variety of readers will find rewarding. □

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How green was the Devonian valley?

Keith Allen

Plant Life in the Devonian.

By Patricia G. Gensel and Henry N. Andrews.

Praeger: 1984. Pp.380. \$29.95, £32.95.

WITHIN palaeobotany, three areas of major and exciting evolutionary change can be distinguished. First, the origin and early evolution of life in the Precambrian; secondly, the rise of vascular land plants in the Upper Silurian and Devonian; and thirdly the origin and evolution of the angiosperms in the Lower Cretaceous.

In 1921 E.A. Newell Arber wrote a small book on Devonian floras. Arber's work supported the growing concept that Devonian land plants included the most primitive of vascular plants, and that this system was one of great evolutionary change, thereby encouraging an increasing number of research workers to study the

Devonian. All the ensuing activity was reflected in the enormous increase in species described, particularly during the past 25 years. But while books have appeared recently on both Precambrian life and angiosperm origins, one or two brief summaries apart there has been nothing on Devonian floras. This new volume by Gensel and Andrews partly satisfies the need for a detailed account of the subject.

Viewing the book as an entity, it seems to me that the authors couldn't decide whether to make it a monograph (though they indicate that it isn't) or a more general work. Much of the book (pp.36–314) contains good, readable descriptions in partial monographic form of selected species, together with discussions of heterospory and early seeds, and more general comments. An early chapter reviews pre-Devonian plants, particularly the microfloral evidence from the Upper Ordovician and Silurian, and later chapters briefly discuss palynology and Devonian floras.

In a volume which is assembled from sifting through an enormous number of papers there is bound to be some lack of

balance, but the descriptive part of the book is unduly biased towards North American and western European species. Past research was indeed more or less confined to these areas, but Devonian floras have been recorded from the Soviet Union since the 1950s, and there has recently been considerable interest in China. The resulting descriptions are usually brief, but in some instances more could have been added at least for floristic comparisons; for example, no mention is made of the important genus *Archaeopteris* occurring in China. Also, in my opinion the authors have not placed enough importance on dispersed spores in the interpretation of Devonian floras. Reference is made to them only in passing in the discussion of heterospory, and in the *in situ* spore studies, yet the sculpture and construction in certain dispersed spores can confidently be used to give further information on age and geographic distributions of floras. Similarly, the section on Devonian floras is too short with the Middle Devonian landscape restricted to less than a page.

There are other points worth questioning. The authors refer to *Cooksonia* as the only undoubted vascular plant found in the Late Silurian, but the evidence is not conclusive that *Cooksonia* is a vascular plant. Even less so is *Steganotheca*, which they include within the vascular family Rhyniaceae, though earlier they mention it within the possible vascular plants. Why, one wonders, aren't *Rhynia gwynne-vaughanii* and *Rhynia major* given separate treatment, rather than being lumped under *Rhynia*, for the "xylem" of *R. major* is clearly very different from that in *R. gwynne-vaughanii*? *Baragwanathia longifolia* is included in the section on Lower and Middle Devonian fossils, when evidence now suggests that the lower *Baragwanathia* horizon is Upper Silurian (to be fair, this latest dating may have been published after the book went to press). It would, too, have been preferable for the descriptions of the rhyniophytes to have preceded rather than followed the trimerophytes; after all, they are less complex, and it seems likely that they gave rise to the trimerophytes. Finally, I am not convinced that a book on Devonian floras should include a detailed account of selected Lower Carboniferous seeds.

In this review I have concentrated on the shortcomings of the book, but, all in all, Gensel and Andrews have done well in compiling such a readable volume. Libraries will doubtless be the main market, though palaeobotanists and taxonomists will be pleased to own a personal copy. For the next edition the authors might consider including more information on palaeoecology, floras and evolutionary trends, which would broaden the potential readership to include third-year undergraduates. □

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Essays into history

Christopher Cullen

The Great Inertia: Scientific Stagnation in Traditional China.

By Wen-yuan Qian.

Croom Helm: 1984. Pp. 155. £15.95, \$24.

MR QIAN is a theoretical physicist who left the Peoples' Republic of China in 1980 after considerable personal suffering during the Cultural Revolution. He is now apparently a permanent exile in the United States, although his wife and daughter are still in China. He has changed disciplines as well as countries; in 1983 he completed an MA in history at Northwestern University, and he is now working for a PhD at the University of Michigan. As Qian himself makes plain, such personal details are not irrelevant to a judgement on his work.

Qian's book is conceived in the form of an attack on what he takes to be basic errors in the work of Joseph Needham. Most readers of this journal will have at least heard of Needham's huge pioneering study of pre-modern Chinese science, technology and medicine under the title *Science and Civilisation in China*, published by Cambridge University Press since 1954. (Seven volumes are planned in thirty tomes, of which eleven have so far appeared.) Qian mounts his attack in three essays which make up the bulk of his book. The first of these argues that the physical sciences in traditional China remained in a state of "stagnation". The second suggests that social factors such as the high degree of ideological control in China, ancient and modern, were responsible for the failure to develop modern science independently in the past, as well as for the failure to make adequate use of it in the present day. The final essay, written from the point of view of a thorough-going mechanistic reductionism, attacks Needham's view that the Whiteheadian organicism which he believes to be inherent in Chinese thought represents a possible way forward for modern science.

Qian's arguments are presented with clarity of expression and the polemical vigour which comes from deep personal involvement. He is passionately in earnest

when he points to the factors which he believes have held back China in the past and continue to hold her back today, and the whole book is imbued with his sense of relief at being in an environment where he is free to speak his mind. I cannot however commend this short book as a significant contribution to scholarship. Qian's three essays try to settle some very broad issues in the history of science and society in East and West. For such attempts to be worth reading, it is essential for their author to base his generalizations on a solid foundation of first-hand knowledge of the full complexities of the historical evidence. Such an acquaintance usually induces a modest tentativeness in coming to conclusions. Qian however is cocksure and dogmatic throughout: all the important questions are simple and he has found the answers to them. Ironically, what he does know about Chinese science seems to have been drawn in its totality from a study of a few of Needham's published volumes. With disarming frankness (p. 19) Qian tells us that he wrote the second essay of his trio before commencing even this very partial exploration. Qian calls his technique of confident seat-of-the-pants generalization "macrohistory". I am afraid it reminds me of what Jonathan Swift called "the art of being deep learned and shallow read".

The root of Qian's problem is that he seems to believe that to be a modern Chinese trained in modern science is ample qualification for writing on the history of Chinese science and society over the past three thousand years. For comparison, how seriously would we take the work of a modern Greek theoretical physicist who published a rapid review of the cosmology of Aristotle? Qian is simply not going to make any impact on the audience he seeks to influence, which is presumably the small but growing group of scholars who study Chinese science from the original source material. His background may be some excuse for being out of touch, but there is no excuse for those who have encouraged him into such damagingly premature publication. □

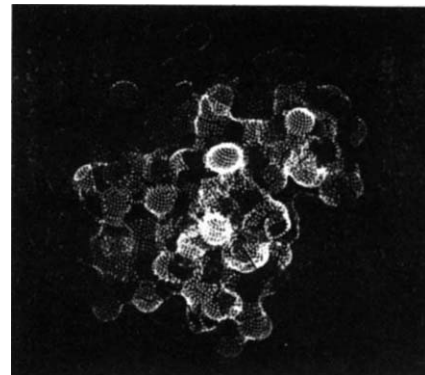
Christopher Cullen is a former Research Fellow of Clare Hall, Cambridge, and has published mainly in the field of ancient Chinese astronomy and mathematics. At present he is Head of Physics at Highgate School, London.



Patterns of agriculture — colour stripping of farmland in Lancaster County, Pennsylvania. The picture is reproduced from *Landprints*, by Walter Sullivan, recently published by Times Books New York (price \$22.50). The book will be reviewed in *Nature* at a later date.

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Questions for the palaeoecologist

Peter D. Moore

Past and Present Vegetation of the Far Northwest of Canada.

By J.C. Ritchie.

University of Toronto Press: 1984.

Pp. 251. \$35, £29.75.

THE most remote corner of Canada, the far Northwest, may seem an unlikely setting for a book about vegetational history. But, as Jim Ritchie points out in his introduction, there are several very good reasons why it should be regarded as a source of interest. In the first place there is its glacial history; rather surprisingly much of the area has escaped major glaciation, certainly for the past half-million years, so we might well expect long, undisturbed profiles of stratified sediments to have accumulated there. Then there is the fact that the region forms the eastern edge of Beringia — the last terrestrial point of contact between the Old World and the New across what are now the Bering Straits. Add to this the wide range of climatic regimes and vegetation types, and the Northwest can be seen to be full of promise for the palaeoecologist.

Ritchie begins by setting the physical and biological scene for the palaeovegetation studies. His emphasis is upon the contrasting environments of the Northwest which result from the gradient between the continental-subarctic climate with early springs in the south, and the arctic-coastal climate with late springs in the north. But the data presented here are not always easy to appreciate simply because there is no map of the locations named. Lack of a map also makes it difficult to interpret some of the intriguing periglacial geomorphology of the region. Take pingos, for example. How does their distribution relate to the limits of glaciation? Are they located only on unglaciated terrain?

A very helpful map of the major vegetation zones is supplied covering not only the Northwest, but also Alaska and eastern Siberia, and is supplemented by outline descriptions of the vegetation types of the area. Detailed lists and phytosociological analyses are given in appendices.

But the main part of the book is given over to the history of vegetation and reconstruction of palaeoenvironments, starting from Tertiary times but concentrating upon the Quaternary. Much of the Tertiary data, which must by now be available as a result of oil exploration, is still hidden from the public gaze and scientific scrutiny.

In view of the unglaciated nature of much of the Northwest terrain, one might hope for long cores of sediment recording Quaternary vegetation history; sadly, this is not the case. Ancient sediments, perhaps dating back about 700,000 years, have been

found in river-bank exposures but no continuous core has yet been obtained.

The last 13,000 years are very well documented from many sites, largely as a result of Ritchie's own work, and the results not only provide a picture of the changing vegetation of the Northwest, but also illustrate many of the problems currently facing palaeoecologists. How do we interpret pollen assemblages for which there is apparently no modern analogue? What are the relative merits and limitations of percentage and influx diagrams? What interpretive allowances must be made for the size of sampling site?

The mass of data and the wealth of

problems described are finally pulled together in a general reconstruction of environmental history. This particular approach results in the combined advantages of a full and detailed picture of the changing vegetation of this interesting area, together with the salutary lessons to be gained from the practical problems encountered in the process. The latter point will make the book valuable even to palaeoecologists whose research interests are far removed from the remote Northwest. □

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Fresh fusion

I.H. Hutchinson

Introduction to Plasma Physics and Controlled Fusion, 2nd Edn. Vol. 1 Plasma Physics.

By Francis F. Chen.

Plenum: 1984. Pp. 421. \$24.50, £23.28.

Fusion: An Introduction to the Physics and Technology of Magnetic Confinement Fusion.

By Weston M. Stacey, Jr.

Wiley: 1984. Pp. 260. \$39.95, £46.20.

WHEN an author publishes a second book covering a specialized scientific topic, as is the case for both books reviewed here, the immediate question that springs to mind is "why?". What is the relationship of the new book to his earlier one? In the case of Francis F. Chen's *Introduction to Plasma Physics and Controlled Fusion* the answer is straightforward: in this second edition of a work originally published in 1974, he plans an expansion of the fusion section into a complete volume, the plasma physics now also taking an entire book. We shall await the appearance of that second volume with interest; meanwhile the present book, apart from some corrections, valuable additions to the chapter on nonlinear effects, and a change to SI units, deviates very little from its predecessor. This is perfectly justifiable since Chen's book has become widely accepted as an excellent introductory text in plasma physics. Its emphasis on intuitive understanding and the copious sketches and diagrams help to make it truly accessible to undergraduates in both physics and engineering.

The author's objective of including where possible all algebraic steps in the development is achieved with surprisingly few tedious sections; however, sometimes this principle leads him to omit the derivation entirely when an outline would have been helpful. It is hard for the student to rectify this failing using other sources because the references are limited mostly to papers whose results are included in figures in the book. Such minor points apart, this

is a deeply satisfying book, full of insight and with a very happy choice of material for a first course in plasma physics. The serious student will want to go further, but this is a good place to start.

The question "why" is more difficult to answer for Weston M. Stacey's *Fusion*. Neither the subtitle, *An Introduction to the Physics and Technology of Magnetic Confinement Fusion*, nor the preface are of much help. The first half of the book consists of an overview of the plasma physics of magnetic confinement. Most of this material, including 18 of the figures, is abstracted from Stacey's *Fusion Plasma Analysis* which appeared only three years ago. Whatever the merits of the earlier book, abbreviating the material for the present work has resulted in a distinctly light-weight treatment. Such an introduction is perhaps appropriate for a non-specialist student; certainly the text is easy to read; but unfortunately it is hard to escape the feeling that simplification has led to superficiality.

The latter half of the book consists of an introduction to the engineering and technology involved in fusion reactor design. Of great merit here is the wealth of physical data included in the form of tables and graphs, which lend a valuable tone of solidity to the engineering issues. Sections on nuclear activation and tritium breeding and handling are particularly welcome. But again the brevity of the treatment and the frequent lack of a proper explanation of the quantities under discussion leaves the reader uncertain of the rigour of any conclusions.

For someone wanting to become broadly acquainted with the various aspects of fusion research, Stacey's book offers a readable introduction. But it is not one which lays a particularly firm technical foundation for detailed research in the field. □

I.H. Hutchinson is an Associate Professor in the Department of Nuclear Engineering at the Massachusetts Institute of Technology.

● Also recently published by Wiley is *Fusion Energy*, by Robert A. Gross, a graduate-level introduction to the principles behind fusion science and technology. Price is \$41.95, £42.90.